

Bioaugmentation strategies for the treatment of pesticide waste streams



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Notation index

2,4-D	dichlorophenoxy acetic acid
3-CA	3-chloroaniline
AMO	ammonium monooxygenase
AMPA	aminomethylphosphonic acid
AOB	ammonium oxidizing bacteria
AOP	advanced oxidation processes
BPS	biopurification systems
CIPC	chloropropham
DGGE	denaturing gradient gel electrophoresis
EPS	extracellular polymeric substance
DNA	deoxyribonucleic acid
HPLC	high performance liquid chromatography
LB medium	Luria Bertani medium
MOB	methane oxidizing bacteria
MM	minimal incubation medium
MMO	methane monooxygenase
MPN	most probable number
PCR	polymerase chain reaction
pMMO	particulate methane monooxygenase
sMMO	soluble methane monooxygenase
VSS	volatile suspended solids

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OUTLINE

Recently, on-farm biopurification systems were developed to prevent pesticide point source contamination of ground- and surface water. However, some pesticides and their degradation products are highly recalcitrant and are not degraded inside a BPS. Two bioaugmentation strategies to enhance the biodegradation potential of biopurification systems were proposed in this work. (i) The usage of the metabolic capabilities of heterotrophic bacteria to improve pesticide degradation. (ii) Employing the pollutant degradation capabilities of the monooxygenase enzyme of methane oxidizing bacteria to co-metabolically degrade pesticides.

In **Chapter 1** we introduce the topic and provide enough information to enable the reader to comprehend the significance of the research questions. At the end of this chapter, the objectives of this thesis are given. In **Chapter 2**, we try to obtain pesticide degrading biofilm cultures and compare their degradation characteristics and microbial community structure with their planktonic counterparts. We also investigate the influence of the biofilm architecture on biodegradation characteristics. In **Chapter 3**, pilot scale BPS systems were inoculated using different types of inocula and substratum materials. We investigate the effect of the different inocula and substratum materials on the removal efficiency of the BPS. In **Chapter 4**, we investigate the spatial and temporal differences in degradation characteristics and microbial community composition inside a BPS. In **Chapter 5** we investigate the cometabolic degradation of pesticides by methane oxidizing bacteria. We also look into the difference between sMMO and pMMO expressing cultures concerning pesticide degradation efficiency and the maximum inhibition concentration. Finally, in **Chapter 6** we discuss the main outcome of our experiments and translate the acquired results into practical guidelines for the installation and practical use of BPS for maximizing the pesticide degrading efficiency of the system. We also propose directions for future research.

CHAPTER 1: INTRODUCTION

1 PESTICIDES: USE, RISKS, REGULATIONS

Concerns over global food security have always been a great incentive to find ways to protect crop production from losses caused by plant pathogens, insects and weeds. Even today, despite agricultural innovations, food losses due to pests, diseases and weeds range from 10 to 90 % with an average of 35 to 40 % for all potential food and fibre crops (Peshin 2002). Up until the 1930s inorganic substances such as sodium chlorate and sulphuric acid or organic chemicals derived from natural sources were used in crop protection. In the 20th century, these inorganic or natural substances have been replaced by synthetic organic molecules.

Pesticides are the only substances intentionally released into the environment to cause a toxic effect to the targeted organisms. When pesticides are applied on the field, only a certain percentage of the applied dose will reach the targeted crop. The remaining fraction will enter the soil, air, surface and groundwater through different pathways and interact with non-target organisms living in the different parts of the ecosystem. Ideally pesticides are only lethal to the targeted pests. Unfortunately there is now overwhelming evidence that some of these substances pose a potential risk to humans and other life and unwanted side effects to the environment (Igbedioh 1991; Forget 1993; Matthews 2006; Damalas et al., 2011; Vergucht et al., 2006). The economic impact of pesticides on non-target species (including humans) has been estimated to be approximately 7,2 billion euro in developing countries (Aktar et al. 2009) and 9.6 billion in the USA (Pimentel 1995).

As a consequence, a shift in usage from highly toxic, persistent and bioaccumulating pesticides to pesticides that degrade more rapidly in the environment and are less toxic to non-target organisms has been established (Vergucht et al. 2006). Furthermore, research has been redirected towards more pest-specific pesticides, a more ecological and biological approach of cropping methods and integrated pest management to minimize the environmental impact of pesticides. In addition, legal frameworks were set up to reduce pesticide usage and spreading. During the past few years new regulations have been implemented in the EU regulating the use of pesticides. The most important regulatory

tool in the EU for plant protection products is Directive 1107/2009 on the placing of plant protection products on the market. Directive 1107/2009 regulates the use of plant protection products and their residues in food. It provides procedures for approval of active substances and plant protection products containing these substances. The Directive states that substances cannot be used in plant protection products unless an appropriate risk assessment has shown that the substance is without unacceptable risk to people or the environment. The assessment is based on efficacy, mutagenicity, carcinogenicity, reprotoxicity, endocrine disrupting properties, persistency and bioaccumulation. The Directive aims to harmonise the authorization process of plant protection products within the EU and to establish a list of active substances that have been shown to be without unacceptable risk (Regulation 540/2011).

Another important regulation is Directive 2209/128/EC establishing a framework for community action to achieve sustainable use of pesticides. Its overall objective is to establish a framework to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticides on human health and the environment and promoting the use of integrated pest management approaches or techniques such as non-chemical alternatives to pesticides. One of the key features of the Directive is that each Member State should develop and adopt its National Action Plan and set up quantitative objectives, targets, measures and timetables to reduce risks and impacts of pesticide use on human health and the environment and to encourage the development and introduction of integrated pest management approaches or techniques in order to reduce dependency on the use of pesticides. Other provisions include compulsory testing of application equipment, training and certification of all professional users, distributors and advisors; a ban on aerial spraying; special measures to protect the aquatic environment, public spaces and conservation areas; minimizing the risks to human health and the environment through handling, storage and disposal. Two other pieces of the European legislation on the subject of plant protection products are also of importance: Regulation 1185/2009 concerning statistics on pesticides which are necessary for assessing policies of the European Union on sustainable development and for calculation relevant indicators on the risks for health and the environment and Regulation 2009/127EC with regard to design, construction and maintenance of machinery for pesticide application.

Other regulations such as the Water Framework Directive 2000/60/EC, Directive 2006/118/EC on the protection of groundwater and Directive 98/83/EC on the quality of water intended for human consumption have had an impact on possibilities for pesticide usage. According to these regulations, the limit for pesticide levels in ground and drinking water in Flanders was set to $0.1 \mu\text{g L}^{-1}$ for any individual active substance and $0.5 \mu\text{g L}^{-1}$ for the total residue concentration. In surface water intended for the production of drinking water concentrations may not exceed $5 \mu\text{g L}^{-1}$ (VLAREM II).

Thanks to these efforts, the use of crop protection agents in Flanders has almost halved in the period 1990-2010. Also the pressure on aquatic life from protection agents has improved 60 % compared to 1990. The biggest part of this improvement can be attributed to the ban of lindane, diuron, paraquat, flufenoxuron and fenoxycarb. Nonetheless, still problems remain for some substances that are commonly used and for which no official standards exist. For example in 2011, ecological reference values were exceeded for diflufenican (a herbicide) flufenacet (a herbicide), carbendazim (a fungicide) and oxadiazon (a herbicide) in 20 or more percent of the sampled measurement locations (Bossuyt et al. 2012).

The distribution of pesticides in freatic groundwater in Flanders in 2006 is presented in Figure 1-1. The Figure shows that official standard levels for groundwater are regularly exceeded. In 56 % of the measurement locations official limits for individual or total pesticide concentration are exceeded. Problem substances in groundwater are VIS-01 (degradation product of chlorthalonil), bentazon, AMPA, atrazine, desthylatrazine, BAM and DMS (VMM (2012)).

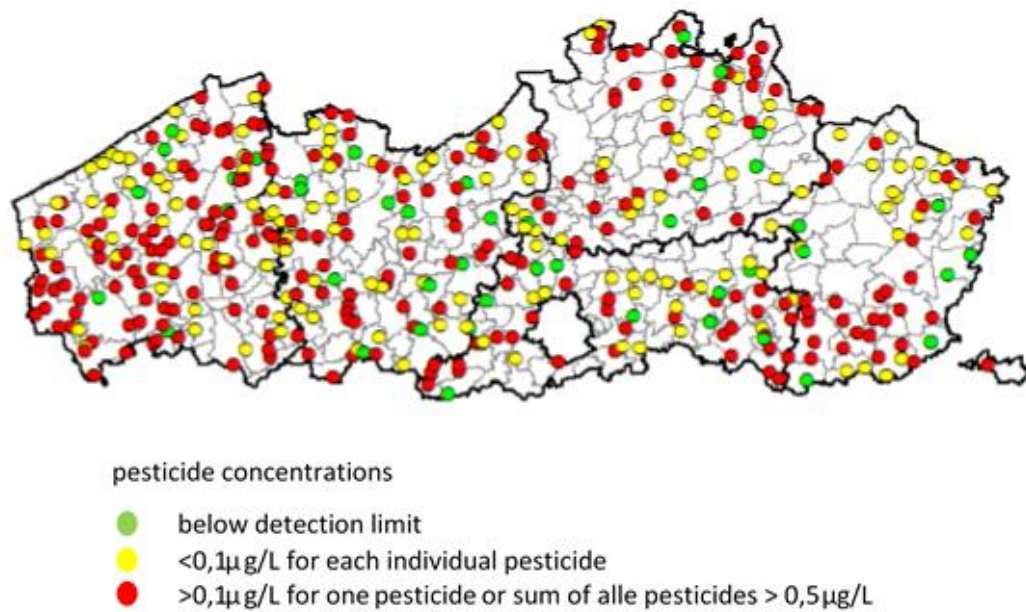


Figure 1-1: Distribution of pesticides (and their degradation products) in the freatic groundwater of Flanders in 2010 (adapted from VMM (2012))

2 ROUTES OF PESTICIDE EMISSION INTO THE ENVIRONMENT

Agricultural food production accounts for about 70 to 80 % of the total pesticide use (Rathore and Nollet 2012). Although pesticide contributions from urban sources to water contamination can also be quite high, especially on a local scale (Blanchoud et al. 2007; Wittmer et al. 2010), pesticide contamination originating from professional agricultural practices are the predominant source.

Pesticides are generally applied on the field where a microbiologically active soil layer is present. The pesticides and their metabolites move through the soil layers whilst degradation and dissipation processes take place. Nonetheless surface and groundwater can be affected due to non-approved use, poor practice, illegal operations and misuse resulting in surface runoff, spray drift and field drainage (Carter 2000). Surface runoff is generated when infiltration capacity and surface storage capacity of soils are exceeded by incoming precipitation. Pesticides in runoff events leave the field either dissolved in runoff water or absorbed to eroded soil particles. Pesticide losses through surface runoff deriving

from agricultural fields are typically less than 0.05% unless extreme 1-2 week rainfalls happen during application time of pesticides (Carter, 2000). Spray drift occurs when wind blows the pesticide solution at application time, and it can cause surface water pollution when spraying is conducted close to water bodies (Carter, 2000). Amounts of pesticide lost from spray drift depend on weather conditions, application methods, technical equipment and type of target crops. Some field monitoring showed that a ground application of a pesticide on arable crops resulted in drift loss ranging from 0.5 to 3.5% of the normal application rate at a distance of one meter from the application area (Carter, 2000). Drainage is responsible for moving excess water from slowly permeable soil, with a shallow groundwater in the field or draining water from fields for cultivation purposes. Pesticide inputs into surface water due to drainage are affected by many factors such as pesticide properties, soil, drainage system, weather conditions and application time. Losses of pesticides due to drainage might be represented by up to 1% of the normal application rate, but typically are less than 1% (Carter, 2000). Volatilization and atmospheric deposition as well as seepage to groundwater are considered to be less significant contamination routes (Rathore and Nollet 2012). These sources of water contamination are called diffuse sources (Table 1-1) and are not clearly localized. They are difficult to identify and predict as they are influenced by various interacting factors such as soil type, weather, pesticide properties and agricultural management practices.

Pesticides can also enter the environment through point sources (Table 1-1). Point source contaminations are mostly related to pesticide handling procedures within the farm yard area. Spills during filling of the spraying equipment, waste water resulting from cleaning of the tank pump and brooms, spills during mixing, leakages of the spray equipment and poor control over left over spray liquids can contribute to direct discharges of pesticides into the environment (De Wilde et al. 2007). Point source contamination can greatly contribute to ground and surface water contamination. One study in Denmark detected concentrations of 2,4-D, dichlorprop, parathion and diquat up to 3800 $\mu\text{g L}^{-1}$ in groundwater that could be attributed to direct contamination due to equipment washing and spilling during the tank filling operations (Helweg et al. 2002). As point source contamination flows can easily contain up to 76 g L^{-1} of active ingredient (Habecker 1989),

small quantities of these concentrated solutions can contaminated a significant water surface above $0.1 \mu\text{g L}^{-1}$ (De Wilde et al. 2007).

Table 1-1: Overview of pesticide emission routes into the environment

diffuse sources	point sources
surface runoff	spillages during tank and sprayer filling
spray drift	spillages mixing
field drainage	leakages of spray equipment
volatilization and atmospheric deposition	poor control over left over spray liquids
seepage to groundwater	washing of spraying equipment

Indeed, the fraction of point source contamination to the total river load of agricultural pesticides has been estimated from 40 up to 70-90 % (Mason et al. 1999; Carter 2000). Given the fact that point sources are largely attributable to operator error or bad practice, equipment faults and physical characteristics of the handling/mixing area, it is considered that point sources can be controlled more easily than diffuse pollution (Rose et al. 2003). Simple measures such as better training of operators and improved design and operation of pesticide handling and wash down areas can greatly minimize the risk of pollution form many point sources. Good results have been obtained by numerous good stewardship initiatives that focused on tank mixing, rinsing, reductions in use and improved spraying practice and resulted in decreased pesticide concentrations found back in surface and ground water (Spiteller et al. 1999; Beernaerts et al. 2003). As a consequence, guidelines for Best Management Practices for pesticide handling have been developed. All handling and storage of pesticides should happen at a fixed place on the farm. The highly contaminated waste water should be collected and treated before disposal (Vaculik et al. 2008).

3 TREATMENT OF PESTICIDE POINT SOURCE CONTAMINATIONS

Several techniques (Physico-chemical as well as biological) are available to treat pesticide waste stream contaminations (Vulto and Beltman 2007). An overview of the techniques that will be discussed in detail in this paragraph can be found in Table 1-2.

Table 1-2: Overview of pesticide point source treatment techniques and their operating conditions (altered from Vulto and Beltman (2007))

	biopurification systems	constructed wetlands	activated carbon filtration	carbo-flo sentinel	advanced oxidation processes
space needed	medium (10 – 20 m ²)	high (10 – 1000 m ²)	low (1 – 5 m ²)	low (1 – 10 m ²)	low (1 – 5 m ²)
technological complexity	low	low	high	high	very high
flow	medium 3000 L y ⁻¹	low 90 m ³ y ⁻¹	high	high (440 m ³ h ⁻¹)	high
can be used in all seasons	no	no	yes	yes	yes

3.1 Physico-chemical treatment methods

The first physico-chemical treatment technique that can be used is *activated carbon filtration*. Activated carbon is a form of carbon that has been processed to have small, low-volume pores that increase the surface area available for adsorption (usually more than 200 m² g⁻¹). The efficiency of the adsorption process depends on the carbon characteristics (particle and pore size, surface area, density and hardness) but also the pollutant characteristics (concentration, solubility of the pollutant and pollutant attraction to the carbon surface). Activated carbon has extensively been tested to treat pesticide waste streams (Kyriakopoulos and Doulia 2006). Problems can occur due to competitive adsorption when a mixture of pesticides is applied or when organic contaminants are

present in the waste water which results in reduced removal rates (Cougnaud et al. 2005). To reduce the high costs of activated carbon filtration, this technique is sometimes preceded by a flocculation step (this system is called ***Carbo-Flo Sentinel***). First flocculation chemicals are mixed with the pesticide waste water. The resulting sludge is sedimented on the bottom of the tank while the supernatant is filtered using a sand filter. Ultimately an activated carbon filter is used to completely remove the remaining pesticides (Maaskant et al. 1992). Another group of treatment techniques generally used to treat pesticide waste streams are ***advanced oxidation processes*** (AOP) (Chiron et al. 2000). In these chemical treatment procedures, organic pollutants are removed by oxidation through reaction with strong oxidants (e.g. hydroxyl radicals). Ozone, hydrogen peroxide, UV light or chlorine can be used to generate radicals. Efficiency can be limited due to the presence of other organic substances. Also transformation products with unknown toxicity can be formed. All these physicochemical treatment methods are highly effective, require limited amounts of space and can treat high flows of pesticide solutions. However they are very expensive and technologically quite complex (Table 1-2).

3.2 Biological treatment methods

Biological treatment could be a cheaper, more effective alternative for on-site remediation of pesticide waste streams, making use of microorganisms that completely degrade polluting compounds. A first biological method is the use of ***constructed wetlands***. Reed fields were first used to reduce the nutrient and carbon content of wastewater, but they can also be used to remove pesticides (Moore et al. 2000; Staerman et al. 2003). The pesticide waste streams are led into the wetlands where the pesticides are removed through biodegradation and sorption processes. Unfortunately, a constructed wetland can have a surface area up to 1000 m² while it can only treat up to 90 m³ of wastewater per year. Therefore the installation of an ***on-farm biopurification system*** (BPS) has also been recommended as a smaller biological system to treat these point source contaminations. In a BPS the pesticide contaminated waste water from an on-farm cleaning and filling place is collected and fed onto a biologically active solid matrix, called a biomix, which typically consists of straw, soil, peat and other organic materials (Castillo et al. 2008). Inside the BPS the pesticides are removed through sorption and biodegradation processes. Pesticide adsorption to the biomix limits the risk for rapid pesticide loss and protects the microflora

from pesticide concentration extremes (Karanasios et al. 2012). Sorption is also a reversible process which allows a sustained bioavailability of the pesticides to the microbial community for subsequent degradation. This system has proven to be low cost, low tech and efficient (Rose et al. 2003). Biopurification systems are used in countries all over the world (Figure 1-2). The best developed and most used biopurification systems are all based on the above mentioned principles and are called the biobed, the phytobac and the biofilter.

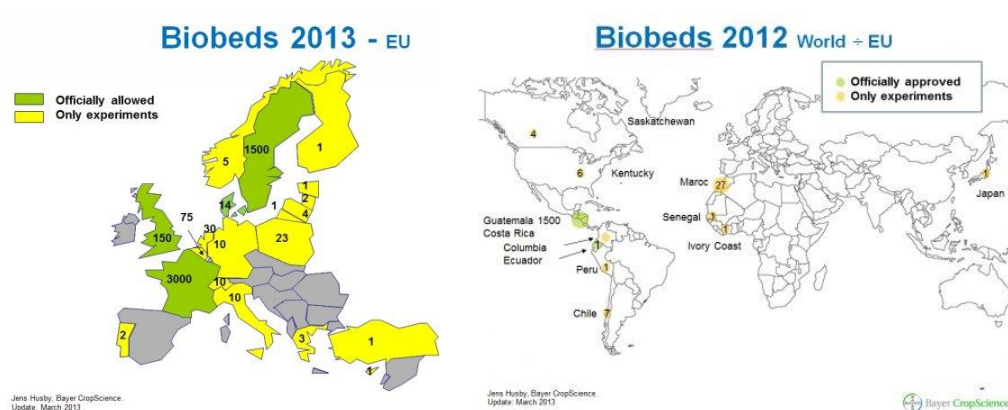


Figure 1-2: Distribution of biopurification systems in the world in 2012-2013 (Jens Huby, Bayer Crop Science, Denmark, www.biobed.org)

3.2.1 Biobed

A biobed is usually a 1m deep pit in the ground which has a 10 cm clay layer at the bottom. This impermeable layer decreases the downward waterflow and increases the pesticide retention time in the biobed. On top of the clay layer a biomixture is added that can readily sorb pesticides while maintaining their bioavailability and optimizing microbial breakdown (Castillo et al. 2008). The biomixture is typically composed of straw, peat and soil although also other locally available materials such as sunflower crop residues, spent mushroom substrate, corn cobs, orange peels or grape stalks can be used (Karanasios et al. 2012). Each component of the biomixture plays an important role in the degradation efficiency and retention of pesticides. Soil is primarily a source of pesticide degrading micro-organisms while straw, peat and other organic substances such as coconut or wood chips are added as a source of nutrients (nitrogen, carbon, phosphor and sulphur) to ensure high microbial activity. Peat contributes to sorption capacity, moisture control, pH

regulation and also abiotic degradation processes (Castillo et al. 2008). Ideally the biomixture is added to the biobed 30 to 90 days before any pesticide solution is added. This allows to initiate the composting process which partially breaks down straw and other substances, which makes it easier to obtain a homogenous mix and makes the biomix more likely to effectively retain and degrade pesticides (De Wilde et al. 2007; Fogg 2007). A grass layer covering the surface of the biobed plays an important role in its temperature and humidity regulation and serves as an indicator of pesticide spillage.

There are two systems: indirect biobeds and direct biobeds. The direct system (Figure 1-4) is equipped with a ramp allowing the farmer to position his spray equipment on top of the biobed where it can be washed and filled over a mesh grid which is positioned on top of the biobed pit. This system is very effective because all spills, drips and waste is collected. The liquid enters the system by gravity so there is no need for pumps (Fogg 2007). However, it can be difficult to keep co-pollutants such as engine oil and grease out of the system (De Wilde et al. 2007). The indirect biobeds (Figure 1-3) are situated next to the sprayer filling area. The pesticide run off is collected and brought on top of the biomix using pumps. This system allows a more controlled distribution of the liquid over the surface area of the biobed.(Fogg 2007).

To achieve the required level of treatment of 1000 liters of pesticide solution per year, biobeds have to be at least 1 m deep in the centre (1.5 m maximum) and have a surface area of 1 m². The total volume of the biobed depends on the total volume of pesticide waste and rinses and the expected annual rainfall. With this system, a maximum amount of 15 000 liters of liquid can be treated (Fogg 2007).



Figure 1-3: An indirect biobed. The sprayer is parked on the handling area with the runoff diverted onto an adjacent biobed (Fogg 2007)



Figure 1-4: A direct biobed system. The sprayer is parked on top of a metal grid. Pesticide waste is intercepted directly by the biobed (Fogg 2007)

3.2.2 *Fytobac*

The biobed concept was picked up by Bayer CropScience France who developed the phytobac, which, in contrast to the biobed, is a completely closed system. It consists of a cistern which is made from concrete or plastic and excess water can only be removed through evaporation. The pesticide containing waste streams can be collected from the filling and cleaning area of the sprayer equipment and stored in a temporary storage tank.

In this way it is possible to regulate the amount of water that is brought on top of the phytobac such that optimal conditions concerning humidity can be achieved. The phytobac has a roof to maximize water evaporation and prevent rain water to interfere with the systems humidity. Per m² of surface area the phytobac can evaporate 300 to 500 L of water per year. This means that phytobacs are generally larger systems in order to avoid complete saturation of the biomix. Drains can be installed to collect the water in a buffer tank and recycle the water several times over the system to increase the systems retention and degradation capacity (Spanoghe et al. 2004). The advantage of this system is that it can process larger volumes of pesticide waste water compared to the biobed system (De Wilde et al. 2007).



Figure 1-5: Phytobac (www.akkerwijzer.nl)

However some practical challenges remain: it is difficult to protect the phytobac from rainfall, to prevent clogging in the pipe circuit and to obtain a homogeneous substratum because mixing of the substratum is not easy (De Wilde et al. 2007).

3.2.3 Biofilter

The basic unit of the biofilter (also known as bio bunk bed) is a 1000 L container, commonly used as batch transport vessel for liquid substances. In most biofilter systems, two or three of these units are stacked on top of each other. A small pump is used to direct the pesticide waste liquid on top of the first biofilter container at the top. To limit the saturation in the container, the containers have an internal drain towards an outflow valve. To stimulate evapotranspiration, a mixture of natural and planted grass (*Miscanthus sp.*), reed (*Fallopia aubertii*) or bamboo can be grown on top of the containers. (Debaer and Jaeken 2006). The plant community also gives an indication on the overall management of the system, can serve as a bio-indicator, temperature regulator, UV radiation shield for the plastic and an esthetic cloak. The use of plants can also stimulate biodegradation by releasing inducers and enzymes that stimulate growth of micro-organisms, stimulates co-metabolic transformations and transforms compounds themselves (De Wilde et al. 2007). However, the used plants should be able to cope with the moist circumstances inside the biofilter and be able to withstand the potentially still high herbicide concentrations that are present in the biofilter environment. (Debaer and Jaeken 2006).



Figure 1-6: Biofilter (www.phytofar.be)

4 BIOAUGMENTATION STRATEGIES TO IMPROVE PESTICIDE DEGRADATION INSIDE A BPS

Inside a BPS pesticides are removed through sorption and biodegradation processes. Pesticide adsorption to the biomix limits the risk for rapid pesticide leaching from the system and protects the microflora from pesticide concentration extremes (Karanasios et al., 2012). Sorption is also a reversible process which allows a sustained bioavailability of the pesticides to the microbial community. Although microbial communities within a BPS might adapt to continuous exposure to pollutants and degrade specific pollutants after a prolonged lag phase, many chemicals or their transformation products remain recalcitrant (von Wiren-Lehr et al. 2001; De Roffignac et al. 2007). A good approach to solve this problem would be to add the degradation potential needed to remove these recalcitrant pesticides.

The required degradation potential can be obtained by supplementing microorganisms with the right pesticide degrading capabilities to the system. In general, microorganisms can use two main catalytic processes to degrade pollutants (Figure 1-7).

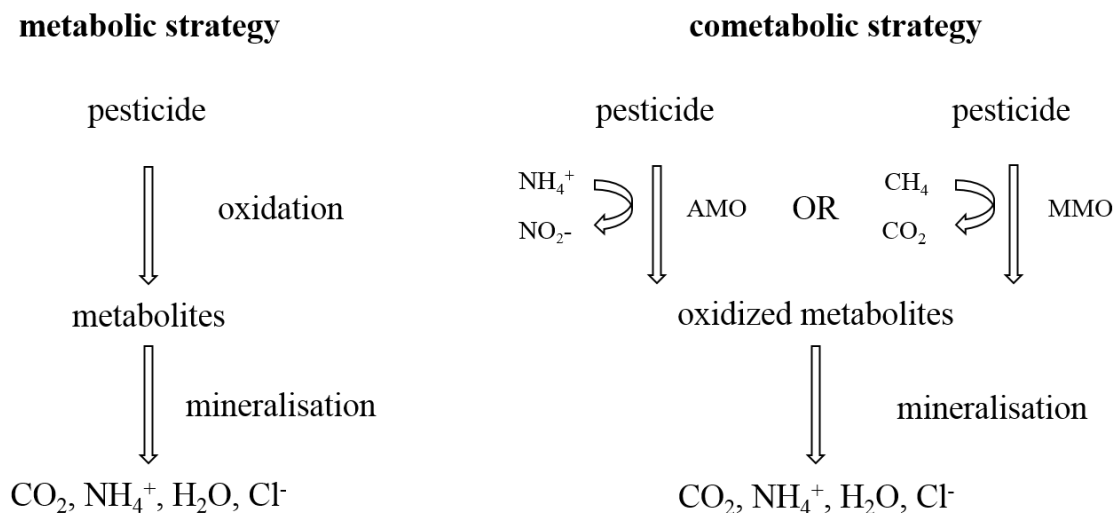


Figure 1-7: Overview of the different removal strategies discussed in this review. Metabolic strategy (left): degradation of pesticides through a metabolic pathway by specific catabolic strains. Cometabolic strategy (right): degradation of pesticides through a cometabolic pathway by ammonium oxidizing bacteria (AOB) or methane oxidizing bacteria (MOB) due to the broad substrate range of their enzyme ammonium monooxygenase (AOB) or methane monooxygenase (MOB). Further mineralization is possible by the associated heterotrophic bacteria.

Firstly, metabolic reactions can be used. These reactions are growth-associated processes and ideally lead to the complete mineralization of the pollutant. In the first part of the review the addition of catabolically potent strains, mixture of strains or an unspecified group of microorganisms to the biomix is discussed. Secondly, some microorganisms can also use cometabolic reactions to degrade pollutants. These are reactions that do not sustain the growth of the responsible microorganism and often lead to the formation of oxidized transformation products (Benner et al. 2013). Two groups of microorganisms e.g. ammonia oxidizing bacteria (AOB) and methane oxidizing bacteria (MOB) which are known to be able to degrade a wide range of pollutants investigated in this part of the thesis. The limitations of their usage are discussed.

4.1 Strategy 1: Metabolic approach: bioaugmentation strategies to enhance pesticide removal inside a BPS

It is generally assumed that increased microbial diversity corresponds to increased catabolic potential and hence to better removal of metabolites and pollutants. According to Dejonghe et al. (2001) this can be achieved in several ways: (1) by adding a catabolically potent strain, mixture of strains or unspecified group of microorganisms to the system. (2) by addition of biodegradation relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms.

- a. Bioaugmentation using a catabolically potent strain, mixture of strains or an unspecified group of microorganisms*

Single isolates or consortia specialized in the degradation of specific pesticides are first isolated from contaminated sites and then used to enhance the pesticide degradation potential of a BPS (Tuomela et al. 1999; Bending et al. 2002). The amount of biomass to be used as inoculum for bioaugmentation is first produced in bioreactors. However, microbial inoculants are often homogeneous cell suspensions that are produced under optimum controlled conditions, so their transference and subsequent establishment into the biomix environment can be problematic. Biotic as well as abiotic stresses may explain the decrease in exogenous microorganisms after addition to the biomix. The abiotic stresses may include fluctuations or extremes in temperature, water content, pH and nutrient availability, along with potentially toxic pollutant levels present in the biomix.

Also competition for limited nutrients, along with predation by protozoa and bacteriophages can play a role (Gentry et al. 2004; Tyagi et al. 2011). Because a biomix is mostly exposed to more than one organic pollutant (different pesticides, co-adjuvants or surfactants), the microbial communities ability to degrade the organic compounds may be inhibited. In such a case it may be advantageous to use multi-component systems such as a microbial consortium that can degrade more than one pollutant ensuring the metabolic diversity and robustness needed for prolonged degradation (Rahman et al. 2002; Diaz 2004). In order to enhance the establishment of the microbial community in the biomix, it could be advantageous to supply the inoculum under the form of a biofilm culture grown on carrying materials, which can then be easily mixed into the total volume of the BPS at start-up. Good results were found for fungal inoculants grown on wood chips (Fragoeiro and Magan 2008) or sawdust (Bastos and Magan 2009). Inocula supplied as biofilms contain cells that are embedded within an EPS matrix. This matrix has been reported to provide protection to the embedded bacteria against a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (Flemming 1993; Singh et al. 2006). Physical and physiological interactions among microorganisms in a biofilm enhance nutrient availability as well as the removal of potential toxic metabolites (Davey and O'Toole 2000). Degrading microorganisms have not been isolated for all known pesticides or their metabolites or sometimes they cannot be cultured (Amann et al. 1995; Runes et al. 2001; van der Meer 2006). Adding **activated materials or an unspecified group of microorganisms** to the biomix could overcome these problems. Activated materials can be defined as materials (soil, straw, peat...) that have been exposed to the pollutants of interest and have developed a degrader population that can eliminate the pollutant. This can have several advantages over using pure cultures or a mixture of pure cultures (Gentry et al. 2004): (1) Naturally developed degrader populations are introduced which contain members or consortia that may not be as effective if they were isolated and applied as pure cultures. (2) The degraders are not cultured outside their natural habitat and therefore do not lose their ability to compete in the environment. (3) Unculturable degraders are included that would

be missed in attempts to isolate and culture the different organisms present in the natural matrix. (4) The presence of a complete catabolic consortium would ensure complete mineralization of the target pesticides. Moreover, this approach would be a simple, low cost, practical and labour-extensive approach for inoculation. However, the pesticide degrading microorganisms inside the biomix are not known. This makes it more difficult to monitor survival and growth of the inoculated microorganisms and to correctly evaluate the used bioaugmentation strategy. Sniegowski and coworkers (2012) found in experiments with lab-scale BPS inoculated with activated top soil that inoculum densities as low as 0.5 vol % provide the biomix with an immediate high linuron mineralization capacity. The microbial community inside the activated materials seemed to withstand and recover rapidly from long periods of sequential unfavorable environmental conditions such as a pesticide supply stop, a period of low temperature and a drought-rewetting period (Sniegowski et al. 2011a). Another approach would be to use the organic matrix from a well-established biopurification system as an inoculation source for a new system. De Wilde et al. (2010b) showed that metalaxyl-primed material, originating from a biopurification system which had been in use for some years and was treated with metalaxyl, significantly increased degradation compared to a soil never treated with metalaxyl. However, the use of activated materials is not always successful. The same study using isoproturon treated soil did not appear to enhance isoproturon degradation. An overview of the recent studies that use bioaugmentation to improve the performance of a BPS is presented in Table 1-3.

Table 1-3: Overview of reported studies on bioaugmentation of pesticide biopurification systems. The table indicates the contaminant that was degraded, the inoculum that was used and the effect of inoculation on reactor performance.

Contaminant	Inoculum	Results of inoculation	Reference
Isoproturon	<i>P. chrysosporum</i>	improved degradation compared to non inoculated reactors	(von Wiren-Lehr et al. 2001)
metalaxyl	<i>C. versicolor</i>	- initiation of degradation of all pesticides on a sterile biomix	(Bending et al. 2002)
terbutylazine	<i>H. fasciculare</i>		
atrazine	<i>S. hirsutum</i>	-better degradation of chlorpyrifos by <i>C. versicolor</i> and <i>H. fasciculare</i>	
diuron			
iprodione			
chlorpyrifos			
Linuron	pesticide primed soil	more rapid and certain establishment of pesticide mineralizing population	(Sniegowski et al. 2011b)
Linuron	pesticide primed soil	inoculated communities are robust to environmental stress (linuron feeding stop, drought period, cold period)	(Sniegowski et al. 2011a)
Linuron	pesticide primed soil	inoculum size of 0.5 vol % is sufficient to achieve effective bioaugmentation	(Sniegowski et al. 2012)
isoproturon	spent biomix	- reduced start up phase	(De Wilde et al. 2010b)
metalaxyl	(treatment history of isoproturon)	-accelerated degradation of metalaxyl - no effect for isoproturon	

b. Addition of genes relevant for biodegradation

Numerous genes or gene clusters conferring pollutant degradation are located on mobile elements such as self-transmissible plasmids. Horizontal gene transfer has been found to play an important role in microbial development and adaptation to

the environment (Ochman et al. 2000). By introducing genetic elements into the indigenous microbial community, its metabolic potential can be increased. Gene mediated bioaugmentation could have several advantages over traditional bioaugmentation: (1) the indigenous microorganisms are already adapted to survive and proliferate in the environment. (2) success is independent of long-term survival of the host strain (Gentry et al. 2004). However, practical implementation can be difficult because not all catabolic genes are located on mobile elements and there is limited knowledge and availability of strains that can degrade pesticides. A thorough review of the use of mobile genes in bioaugmentation can be found in Top et al. (2004). Very few studies have evaluated the introduction of degradative bacteria with mobile genetic genes into wastewater treatment systems and the success rate of these efforts has been limited (McClure et al. 1989; Ravatn et al. 1998; Ghyoot et al. 2000; Springael and Top 2004; Ikuma and Gunsch 2013; Tsutsui et al. 2013). To the best of our knowledge, this approach has not yet been tested in a BPS environment.

4.2 Strategy 2: Cometabolic degradation of pollutants using nitrifying and methane oxidizing bacteria

Cometabolism is another process that can be used for removal of contaminants in wastewater. In this process, non-specific enzymes degrade pollutants without the respective microorganism gaining energy or carbon for microbial growth with this process. Next to white rot fungi, which grow on lignocellulosic substrates and can cometabolically degrade pesticides (for an overview: see Rodriguez-Rodriguez et al. (2013)) and heterotrophs that can cross feed on the original molecule or its metabolites (Pelz et al. 1999), both nitrifying and methanotrophic bacteria possess an enzyme with a broad substrate specificity that can degrade micropollutants.

a. Cometabolic degradation using ammonia-oxidizing bacteria

Ammonia-oxidizing bacteria (AOB) can convert ammonium into nitrite (nitration) by two sequential enzymatic processes (Kowalchuk and Stephen 2001). First ammonia (NH_3) is oxidized to hydroxylamine (NH_2OH) using the membrane bound enzyme ammonium monooxygenase (AMO). Subsequently NH_2OH is converted into nitrite (Hooper et al. 1997). AMO has been found to be able to

cometabolically degrade a wide range of pollutants, such as trichloroethylene (Yang et al. 1999; Kocamemi and Cecen 2005; Kocamemi and Cecen 2007; Kocamemi and Cecen 2010a), 1,2-dichloroethane (Kocamemi and Cecen 2009; Kocamemi and Cecen 2010b), chloroform (Arp and Stein 2003), chlorobenzenes (Arp and Stein 2003) and of several pharmaceuticals such as beta blockers (Sathyamoorthy et al. 2013) and 17 α - ethinylestradiol (Vader et al. 2000; De Gusseme et al. 2009).

Removal of pollutants in nitrifying bioreactors has mostly been studied in membrane bioreactors (MBR) (Vader et al. 2000; Batt et al. 2006; De Gusseme et al. 2011) but also other systems such as continuous flow nitrifying biofilm reactors (Kocamemi and Cecen 2009) and packed bed reactor (Frasconi et al. 2014) exist. In on-farm BPS, manure could be dosed to the BPS serving as the inexpensive ammonium source allowing proliferation of AOB inside the biomix. Despite the advantages of using AOB to cometabolically degrade pollutants, some problems limit the usage of this process. First of all, both the growth substrate and the cometabolic substrate compete for the same active site of the MMO enzyme which results in competitive inhibition between the growth substrate and the cometabolic substrate. This leads to reduced oxidation rates of both substrates (Alvarez-Cohen and Speitel 2001). Secondly, the intermediates of the cometabolic degradation can be toxic resulting in decreased AMO activity and decreased cell viability (Rasche et al. 1991). Finally, to allow the development of the slow growing nitrifying bacteria, long solid retention times are needed.

b. Cometabolic degradation using methane oxidizing bacteria

Many natural but also anthropogenic sources including natural wetlands, rice paddies, ruminants, lakes and oceans, soils, landfills, oil and gas recovery operations and even termites produce methane gas. As with nearly all other naturally produced organic molecules on Earth, there are also microorganisms capable of using methane as their sole source of carbon and energy. These microbes (called methanotrophs) are ubiquitous and for the most part aerobic (Dalton 2005). A multicomponent enzyme system – methane monooxygenase (MMO) – is responsible for their unique ability to use methane for growth. MMO

has two distinct forms: soluble MMO which is located in the cytoplasm (sMMO) and membrane associated particulate MMO (pMMO). Although both sMMO and pMMO oxygenate methane to methanol, they show no similarity in the amino acid sequence of their protein components. Methane is the preferred substrate, but pMMO as well as sMMO have been found to be nonspecific. sMMO has a broader substrate range compared to pMMO and can oxidize alkanes up to C-8, as well as ethers, cyclic alkanes and aromatic hydrocarbons (Colby et al. 1977; Burrows et al. 1984; Amann et al. 1995). pMMO has a narrower substrate range, being able to oxidize alkanes up to C-5 but not being able to oxidize aromatic compounds (Burrows et al. 1984). Methanotrophs have also been shown to be able to oxidize priority pollutants such as halogenated aromatics and biphenyls via sMMO activity (Green and Dalton 1989) or halogenated hydrocarbons via pMMO activity (Lontoh and Semrau 1998; Han et al. 1999; Lee et al. 2006). It has been found that sMMO expressing cells can degrade chlorinated ethenes at much faster initial rate than pMMO expressing cells (Lontoh et al. 2000). However, most known methanotrophs can express pMMO while sMMO is expressed mainly by type II and type X methanotrophs under copper deficient conditions (Hanson and Hanson 1996; Choi et al. 2003; Semrau et al. 2010). This requirement for the absence of copper to induce the production of sMMO can be a handicap for bioremediation. Mutant strains of *M. trichosporium* OB3b which lack the ability to express pMMO but are able to express sMMO constitutively even when copper concentrations are up to 12 μM have been developed and successfully used in bioreactors (Phelps et al. 1992; Tschantz et al. 1995; Fitch et al. 1996). However, successful application of these strains in practice is limited due to the particular nutritional requirements (such as vitamins and yeast extract) to maintain degradation activity. Although sMMO expressing methanotrophs can degrade individual chlorinated ethenes at higher rates (Oldenhuis et al. 1991; Lontoh et al. 2000), such degradation may ultimately be limited due to the greater product toxicity associated with sMMO activity compared to the same cell expressing pMMO (Alvarez-Cohen and McCarty 1991). Also, Lee et al. (2006) reported that at concentrations of VC, t-DCE and TCE greater than 10 μM each, methanotrophs expressing pMMO have a

competitive advantage over cells expressing sMMO due to higher growth rates. Despite this growth advantage, pMMO expressing cells degraded less of the added pollutants than sMMO expressing cells. However, when pollutant concentrations were increased to 100 μ M, pMMO expressing cells grew faster and degraded more of the added pollutants in a shorter amount of time. Recent studies show that pMMO expressing methanotrophs are also able to degrade more complex aromatic structures such as bentazon, sulfamethoxazole and benzotriazole although product toxicity levels and the degradation efficiency are lower compared to sMMO expressing methanotrophs. (Benner et al. 2014). All these findings suggest that the relative rates of growth substrate and pollutant degradation are to be considered when determining which form of MMO should be considered for pollutant degradation.

4.3 Conclusion

The degradation capacity of these systems can be improved in two different ways: by a metabolic or cometabolic strategy. The addition of pesticide degrading strains or consortia and the transfer of catabolically potent genes to the indigenous microbial community has been successfully applied and tested in a BPS environment. The use of ammonia and methane oxidizers in small scale on farm biopurification systems would be a novel strategy. Thanks to their broad oxidizing capabilities, these microorganisms have the potential of providing the first step necessary for the subsequent complete mineralization of these pollutants by heterotrophs. Nevertheless, some problems concerning competitive inhibition between growth substrate and pollutant for the active enzyme as well as toxicity of the pollutant or its intermediates need addressed. Provided these challenges can be overcome, AOB as well as MOB have an enormous potential for use in biodegradation.

5 OBJECTIVES AND OUTLINE

Recently, on-farm biopurification systems (BPS) were developed to prevent pesticide point source contamination of ground- and surface water. However, some pesticides and their degradation products are highly recalcitrant and are not degraded inside a BPS. In this thesis bioaugmentation as a strategy to enhance the biodegradation potential of BPS is investigated. Two bioaugmentation strategies are proposed: (i) The usage of the metabolic capabilities of heterotrophic bacteria to improve pesticide degradation. (ii) Employing the pollutant degradation capabilities of the monooxygenase enzyme of methane oxidizing bacteria to co-metabolically degrade pesticides.

The objectives of this thesis were:

- (1) To compare the use of an enrichment strategy with the use of a biofilm enrichment strategy to obtain chloropropham degrading species and consortia and to compare the obtained planktonic and biofilms cultures in terms of their degradation rate as well as their bacterial community composition (chapter 2).
- (2) To evaluate the use of a chloropropham degrading biofilm culture and a single strain culture as an inoculant in a pilot scale bioreactor using two different substrata (plastic chips and biomix) (chapter 3).
- (3) To evaluate the influence of the BPS depth on the size and composition of the chloropropham and 3-chloroaniline catabolic populations and to investigate spatial and temporal variability in the composition of the chloropropham and 3-chloroaniline catabolic communities during reactor operation (chapter 4).
- (4) To investigate if and at which concentrations pesticides can be co-metabolically degraded by methane oxidizing bacteria (MOB) and to examine the influence of copper addition on the microbial community composition, the pesticide degradation efficiency and the maximum inhibition concentration (chapter 5).

Chapter 6 is the general conclusion of this work, where the results obtained are evaluated in the framework of the research objectives. Implementation of these two strategies to bioaugment BPS is discussed while critically examining the technical limitations and scientific challenges that need to be addressed prior to successful implementation. Further research perspectives are discussed, together with the opportunities and challenges of using metabolic and co-metabolic strategies to enhance pesticide removal in on-farm biopurification systems.

CHAPTER 2:

PLANKTONIC VERSUS BIOFILM CATABOLIC COMMUNITIES: IMPORTANCE OF THE BIOFILM ARCHITECTURE FOR SPECIES SELECTION AND PESTICIDE DEGRADATION

ABSTRACT

Chloropropham degrading cultures were obtained from sludge and soil samples through two different enrichment techniques i.e. (i) planktonic enrichments in shaken liquid medium and (ii) biofilm enrichments on two types of solid matrixes (plastic chips and gravel). DGGE fingerprinting showed that planktonic and biofilm cultures had a different community composition depending on the presence and type of the added solid matrix during enrichment. This was reflected in the unique chloropropham degrading species that could be isolated from the different cultures. Planktonic and biofilm cultures also differed in chloropropham degrading activity. For biofilm cultures slower chloropropham removal was observed, but with less build-up of the toxic intermediate 3-chloroaniline. Disruption of the biofilm architecture resulted in degradation characteristics shifting towards those of the free suspensions, indicating the importance of a well established biofilm structure for good performance. These results indicate that biofilm mediated enrichment techniques can be used to select for pollutant degrading microorganisms that like to proliferate in a biofilm and that cannot be isolated using the conventional shaken liquid procedures. Furthermore, the influence of the biofilm architecture on the pesticide degradation characteristics suggests that for bioaugmentation the use of biofilm catabolic communities might be a proficient alternative to using planktonic freely suspended cultures.

Chapter redrafted after:

Verhagen, P., De Gelder, L., Hoefman, S., De Vos, P. and Boon, N. (2011) Planktonic versus biofilm catabolic communities: importance of the biofilm for species selection and pesticide degradation. *Applied and Environmental Microbiology* **77**, 4728-4735.

1 INTRODUCTION

The fate of pesticides in nature is of great environmental concern due to their toxic effects on human and animal health and their negative impact on biodiversity and overall environmental quality (Matthews 2006). Since the synthesis and commercialization of carbamate pesticides 60 years ago, chloropropham ([Isopropyl N-(3-chlorophenyl) carbamate] or CIPC) has become one of the most commonly used potato sprouting suppressants. It can also be used as a selective systemic herbicide for the control of many annual grasses and some broad-leaved weeds in various food and non-food crops (Chapalamadugu and Chaudhry 1992; Tomlin 2006). The compound is slightly to moderately toxic to mammals, birds and aquatic organisms and is moderately persistent in the environment: soil half-lives of 65 days at 15 °C or 30 days at 29 °C have been reported (Tomlin 2006). Because of its resistance to hydrolysis and oxidation, bacterial degradation is the dominant elimination pathway in the environment (Wolfe et al. 1978). It leads to the formation of the toxic intermediate 3-chloroaniline which can be mineralized further (Kaufman and Kearney 1965).

Bioremediation has generally been considered as a cost-effective and efficient method for mineralization and detoxification of various pollutants and harmful xenobiotic compounds. In the case of pesticides, the use of on-farm biopurification systems to treat point source contamination has been proposed (De Wilde et al. 2007). The pesticide degradation capacity of these systems can be enhanced using specialised pesticide degrading bacterial isolates or consortia. Therefore, many efforts have been undertaken to isolate mono –and mixed cultures capable of degrading pesticides. In most isolation strategies, enrichments are carried out in shaken liquid medium, which favours bacteria that grow well in suspension. However, in nature, most bacteria grow aggregated with each other and with solid surfaces (Parsek and Fuqua 2004). As biofilms are the predominant mode of bacterial life (Costerton et al. 1995; Moons et al. 2009), the use of shaken liquid medium enrichment might be an important bias because bacteria that like to proliferate in a biofilm and grow very poorly in suspension may be outcompeted using this selection procedure. By adding a solid support to the medium during the enrichment process and transferring support-bound organisms only, biofilm-forming micro-organisms might be favoured over freely living cells.

Furthermore, the cells in a biofilm are embedded within an EPS matrix which has been reported to provide the embedded bacteria protection against a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (Flemming 1993; Singh et al. 2006). Physical and physiological interactions among microorganisms in a biofilm enhance nutrient availability as well as the removal of potential toxic metabolites (Davey and O'Toole 2000). Indeed, recent studies suggest that biofilm-mediated bioremediation can be a proficient alternative to remediation using planktonic microorganisms (Singh et al. 2006).

Despite these advantages, only few attempts have been made to isolate pollutant degrading microorganisms that form a biofilm (Bastiaens et al. 2000; Breugelmans et al. 2007). The present study was designed to investigate whether addition of a solid matrix during enrichment enhances biofilm formation and influences its biodegradation properties. The influence of different solid matrixes on the community composition and degradation characteristics of the obtained enrichment cultures was investigated and compared to conventional shaken liquid enrichment. Finally experiments were set up to investigate the involvement of the biofilm architecture on the observed results.

2 MATERIAL AND METHODS

Media and culture conditions

The minimal incubation medium (MM) used in this study was based on Stanier medium (Stanier et al. 1966). It contained 1419.6 mg Na_2HPO_4 , 1360.9 mg KH_2PO_4 , 300 mg $(\text{NH}_4)_2\text{SO}_4$, 98.5 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.88 mg $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 2.78 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.69 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.15 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.38 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.24 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.12 mg $(\text{NH}_4)_6\text{Mo}_{24} \cdot 4\text{H}_2\text{O}$ and 3.2 mg Na_2EDTA per liter of distilled water. The liquid minimal medium (MM-CIPC) was supplemented with an appropriate concentration of chloropropham (technical grade, purity > 98 %, Certis Europe, Belgium). Solid medium MM agar plates were obtained by adding 15 g noble agar (Sigma-Aldrich Chemie, Germany) per liter of MM. Because of the low solubility of chloropropham, spray plates were made based on Kiyohara et al. (1982) by spraying an ethereal solution of chloropropham (5 %, wt/vol) uniformly over the surface of the agar plates. Diethylether immediately vaporized from the surface of the plates and a white thin layer of

chloropropham remained on the entire surface which could be used by chloropropham degrading bacteria as the sole carbon source. LB medium containing 10 g of peptone (Applichem, Germany.), 5 g yeast extract (Applichem, Germany) and 5 g of NaCl in 1 liter of distilled water was used as a rich medium. When chloropropham degraders were grown on rich medium, 80 mg/L chloropropham was added to the medium to make sure the bacteria would not lose their degradation capacity in case of plasmid-encoded degradation genes. Solid LB medium was obtained by adding 15 g/L of agar. All glassware and media were sterilized by autoclaving (121 °C, 15 min).

Enrichment procedure

Enrichment cultures were set up from activated sludge of a potato-processing company (Waregem, Belgium) and from soil (sandy loam, pH = 7.2 ± 0.2 , moisture content = $8.28 \% \pm 0.05 \%$ organic content = $2.05 \% \pm 0.12 \%$) of a potato storage facility that has been exposed for more than 30 years to high doses of chloropropham (Kruishoutem, Belgium). Three erlenmeyers (250 mL capacity) containing 20 mL of activated sludge (4.5 g dry weight/L) in 80 mL of MM-CIPC medium (final concentration of 50 mg/L chloropropham) were used to initiate the first series of enrichments. To the first erlenmeyer, no solid support was added, but the second and the third erlenmeyers were supplemented with 50 g of gravel (average size 9.5 mm, surface area: $150 \text{ m}^2/\text{m}^3$) and 6 plastic chips (Biofilm-Chip M, diameter: 48 mm, surface area: $1400 \text{ m}^2/\text{m}^3$, AnoxKaldnes, Sweden) respectively, in order to select for chloropropham degrading microorganisms that could grow as a biofilm. The second series of three enrichments was initiated similarly by adding 5 g of sieved soil ($< 2 \text{ mm}$) to 100 mL MM-CIPC medium (final concentration of 50 mg/L chloropropham). Sterile controls to monitor abiotic losses of chloropropham during the enrichment were made by autoclaving the soil 3 times. All enrichments and controls were incubated at 28 °C on a rotary shaker (120 rpm). Chloropropham concentrations were monitored using HPLC and when the concentration of chloropropham in the medium fell below the quantification limit (0.1 mg/L), a new erlenmeyer flask containing 100 mL of MM-CIPC medium (final concentration of 50 mg/L chloropropham) was inoculated with 2 mL of the enrichment culture in case no solid support was present in the enrichment culture. In case a solid support was present in the enrichment, the solid support itself was transferred to fresh MM-CIPC medium. All chloropropham enrichments cultures were

enriched for 10 weeks (corresponding to 10 medium refreshments) before plating. In Figure 2-1 a schematic overview of the procedure is given.

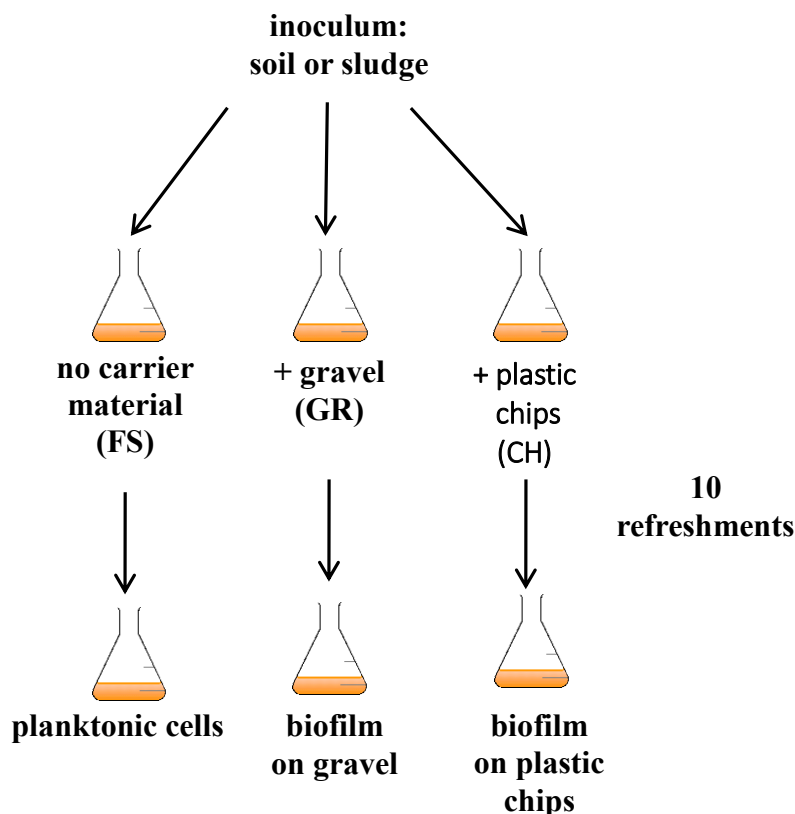


Figure 2-1: Schematic overview of the enrichment procedure

Isolation procedure

After 10 medium refreshments, the biofilms were removed from their solid support and vigorously vortexed in 10 mL of MM medium (2400 rpm). 100 μ L of each free suspension and biofilm enrichment were spread on separate chlorophoram spray plates, which were incubated aerobically at 28 °C for 5 days. Because of the small size of the colonies present on the different plates, no morphological differences between them could be distinguished on the film plates. Therefore, colonies were picked up and purified on LB-agar plates containing 80 mg/L chlorophoram. After a 2 day incubation at 28 °C, single colonies with a different morphology were picked up from each plate and the ones that were able to degrade chlorophoram in liquid MM-CIPC were regarded as chlorophoram assimilating bacteria.

BOX-PCR

The template for PCR amplification was obtained by extracting total genomic DNA from the pure cultures using a procedure described by Boon et al. (2000). The BOX-PCR was performed to distinguish identical isolates (Koeuth et al. 1995).

Fatty Acid Methyl Ester Analysis

Pure cultures were identified using fatty acid methyl ester analysis (FAME). The cultures were grown aerobically on Trypticase Soy Broth (TSB; BBL, Becton Dickinson Microbiology Systems, USA) with 1.5% Bacto Agar (Difco) for 24 h at 28°C. Fatty acid extraction and analysis was performed as described by Sasser et al. (Sasser 1990) and Dawyndt et al. (Dawyndt et al. 2006) respectively. An identification based on the resulting profiles was obtained with the Sherlock Microbial Identification System using the TSBA database version 5.0 (MIDI; Microbial ID, USA). These identifications were confirmed by comparing the profiles qualitatively and quantitatively with an in-house database containing over 70,000 fatty acid profiles.

DNA Extraction and 16S rRNA Gene Sequence Analysis

Pure cultures were identified by extracting bacterial genomic DNA from each isolate as described by Pitcher et al. (Pitcher et al. 1989). PCR amplification, purification with the Nucleofast® 96 PCR system (Millipore) and subsequent sequence generation of the 16S rRNA gene was performed as described by Heyrman and Swings (Heyrman and Swings 2001). Sequencing products were purified with the BigDye XTerminator® Purification Kit (Applied Biosystems) according to the manufacturer's instructions. The sequences, with a length of approximately 1500 bp were analyzed using a 3130 XL Genetic Analyzer (Applied Biosystems, USA) and an identification was obtained as described by Coorevits et al. (Coorevits et al. 2008) using the BioNumerics 5.1 (Applied Maths, Belgium) software. All sequences were deposited in EMBL with accession numbers FR682925 to FR682939.

Bacterial community analysis

The template for PCR amplification was obtained by extracting total genomic DNA from the enrichment cultures using a procedure described by Boon et al. (Boon et al. 2000). A

100 μ L aliquot of the crude extract was further purified with the Wizard DNA clean-up kit as described by the manufacturer (Promega, USA). 100 ng of purified genomic DNA was used to amplify 16 S rRNA gene fragments with an Applied Biosystems 2720 Thermocycler using the primers PRBA338fGC and P518r (35 cycles) (Muyzer et al. 1993). Denaturing gradient gel electrophoresis (DGGE) was performed with a denaturing gradient ranging from 45 to 60 % (Boon et al. 2002). The obtained DGGE patterns were subsequently processed using BioNUMerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated based on the Pearson correlation coefficients. In order to graphically represent the evenness of the bacterial communities, Lorenz distribution curves were set up based on the DGGE profiles as previously described (Marzorati et al. 2008). Community organisation values (Co) were defined as $100 \times \text{gini-coefficient}$.

Batch degradation experiments

Enrichment cultures and isolates were grown in MM medium with 50 mg/L chloropropham. After two days another 200 mg/L chloropropham was added to the cultures. When chloropropham was completely mineralised, the biomass concentration was determined and expressed as the volatile suspended solid concentration. To examine the chloropropham removing efficiency of each culture, a batch test was set up in triplicate in 250 mL autoclaved glass erlenmeyers containing 100 mL minimal medium with a final concentration of 25 mg/L chloropropham and 50 mg VSS/L culture. To confirm that the removal of chloropropham was biologically driven, a similar batch test was set up by incubating the erlenmeyer flasks with heat inactivated biomass (20 min, 121 °C). Liquid samples for HPLC analysis were taken at 30 minutes intervals. All tests were done in triplicate.

Influence of the biofilm architecture on chloropropham degradation characteristics

To evaluate the influence of the biofilm architecture on the chloropropham removal and accumulation of 3-CA, the biofilm architecture was disturbed using two strategies. The first strategy involved removing the biofilm biomass from its solid support and rigorously vortexing the formed flocs. The removing of the biofilm from its solid support was done using a syringe and needle to rinse the solid support with minimal medium. During

vortexing the solid support was no longer in the culture. In the second strategy, the flocs biomass was regrown using minimal incubation medium with added chloropropham in the absence of a solid support. The first technique resulted in a suspended culture, but most cells were still associated with a biofilm as pieces of biofilm were still visible under the microscope. The second technique resulted in the formation of a free suspension culture. These two types of suspended cultures were used as inocula for biodegradation experiments. For each biodegradation experiment, a unique inoculum generated using one of the above procedures was used.

Viability test

Live/dead staining was used to investigate the effect of vortexing on the viability of the biofilm biomass. The biomass was stained using a live/dead Backlight Bacterial Viability Kit (Molecular Probes, Eugene CA, USA). 10 pictures were randomly taken before and after vortexing of the biofilm using a fluorescence microscope. Live/Dead ratio was determined using an image processing program (ImageJ, National Institute of Health). Vortexing did not seem to have any effect on the biofilm biomass viability.

Analytical methods

Supernatants of bacterial cultures were analyzed by reverse-phase HPLC after the cells were removed by centrifugation at 5000 g for 10 minutes.

Chloropropham and 3-chloroaniline were analysed using a HPLC system (HP Agilent 1100 series) equipped with a G1322A degasser, a G1311A quaternary pump, a G1313A autosampler, a G1314A variable wavelength detector, a G1316A column compartment and HP Chemstation software. A Gracesmart RP-18 column (250- by 4.6-mm inner diameter, 5- μ m particle size; Grace, USA) was used. The mobile phase consisted of CH₃OH/0.1 % H₃PO₄ (60/40) with a flow rate of 1.0 mL min⁻¹ and the UV detector was set to 240 nm. Quantitative determination of chloropropham and 3-chloroaniline was done using an external standard ranging from 0.1 to 60 mg/L. The quantification limit was 0.1 mg/L.

Analysis of Cl⁻ was performed using a Methrohm 761 Compact Ion Chromatograph (Methrom, Herisau, Switzerland) equipped with a conductivity detector. The operational

parameters were as follows: column, Metrosep A supp 5; eluent, 1.06 g l⁻¹ Na₂CO₃; flow, 0.7 ml min⁻¹; sample loop 20 µl.

Statistical analysis

To test for significant differences between means a one-way ANOVA test was performed using SPSS (IBM SPSS v21). When p-values were lower than 0.05 differences were considered to be significant.

3 RESULTS

Characterisation of the chloropropham degrading microbial communities

All sludge and soil enrichment cultures (free suspension, gravel and chips) were able to completely remove 50 mg/L chloropropham in less than 48 h from the fifth medium refreshment on, and after 10 enrichments, a biofilm was clearly visible on all added carrier materials while in the free suspension cultures, no biofilm formation could be observed.

After 2, 5, 7 and 10 medium refreshments, the bacterial community composition of the different enrichment cultures was compared using 16S rRNA gene-based community fingerprinting (Figure 2-2 A,D). To determine the information content of the banding patterns in terms of structural diversity, the samples were analysed by clustering (Figure 2-2 B,E). The DGGE patterns of both sludge and soil enrichment cultures constituted two very distinctive clusters reflecting the early and late stages of the enrichments. After 10 enrichments, the cluster analysis revealed a clearly different community structure between the free suspension and biofilm enrichments with similarities between the free suspension and gravel, and the free suspension and chips enrichments of 52.3 ± 2.3 % and 62.4 ± 7.2 % respectively for the sludge enrichments and 31.2 ± 15.1 % and 74.9 ± 0.0 % respectively for the soil enrichments. Based on the DGGE profiles of the final enrichments, a graphical representation of the structure of the bacterial community was made using Lorenz evenness curves (Figure 2-2 C,F). From the Lorenz curves community organisation values (Co) were calculated, representing a numerical value for the species evenness. The free suspension enrichments showed a less evenly distributed community compared to the biofilm enrichments. To compare species richness among the different final enrichment cultures, R values representing the number of bands in the DGGE profile were calculated. The

number of bands was more than 50 % lower and 21 to 34 % lower for the free suspension cultures compared to the gravel and chips enrichment cultures for the sludge and soil enrichment respectively.

Isolation and identification of chloropropham metabolising bacteria

After plating the tenth and final enrichment cultures on MM agar with chloropropham as the sole source of carbon, a total of 127 colonies were selected from the different plates and tested for their ability to degrade chloropropham (Table 2-1). Out of these 127 isolates, 81 could use chloropropham as the sole source of carbon. Each of these strains was able to completely remove chloropropham at a removal rate between 485 ± 26 and 618 ± 27 mg CIPC. g⁻¹ VSS. d⁻¹. During chloropropham removal, a maximum of 3.9 mg/L 3-CA was temporarily formed, but this intermediate was metabolised subsequently. Remarkably, when grown in the presence of a solid support, none of the isolated strains formed a biofilm.

Clonally identical isolates were discriminated using BOX-PCR. Fifteen unique BOX-patterns could be distinguished. Representatives of each group of strains showing identical BOX-PCR genomic patterns were identified by 16S rRNA gene analysis. The 15 different strains as well as the enrichment from which they were isolated and their relative abundance inside each enrichment are presented in Table 2-1. Concerning the sludge enrichment cultures, four out of five species could be isolated from the biofilm enrichment cultures as well as from the free suspension culture, although *Pseudomonas* sp. R-41382 could only be isolated from the gravel enrichment culture. Regarding the soil enrichment cultures there was a more distinct difference concerning the species that could be isolated from the free suspension and the biofilm enrichment cultures. Three species (*Diaphorobacter* sp. R-41385, *Stenotrophomonas* sp. R-41388 and *Achromobacter* sp. R-41391) could only be isolated from the free suspension culture while five species (*Delftia* sp. R-41393, *Pseudomonas* sp. R-41394, *Pseudomonas* sp. R-41394, *Achromobacter* sp. R-41395 and *Pseudomonas* sp. R-41398) could only be isolated from the biofilm enrichment cultures. Two species (*Pseudomonas* sp. R-41389 and *Pseudomonas* sp. R-41390) were found in the biofilm enrichment cultures as well as in the free suspension culture.

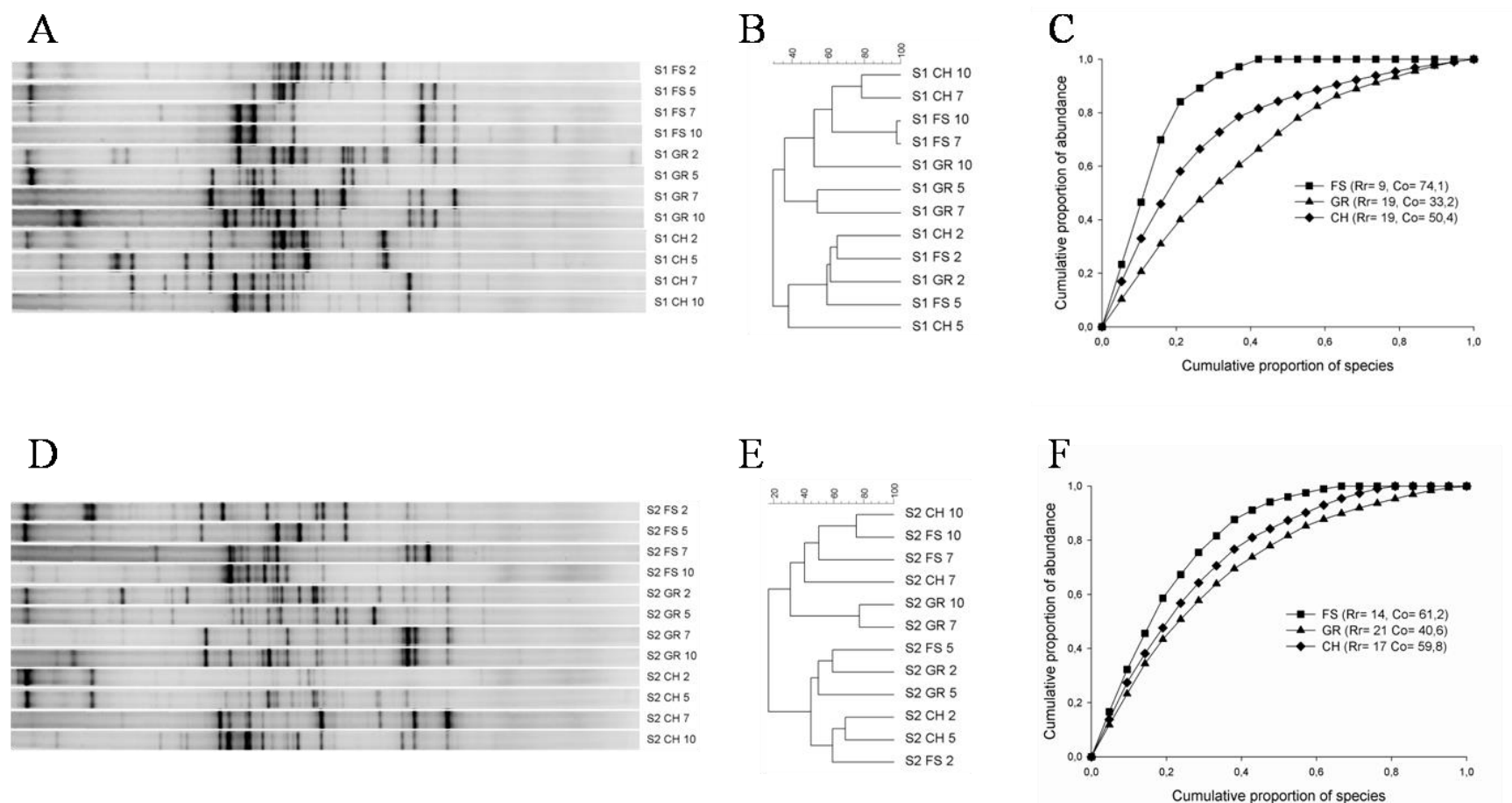


Figure 2-2: DGGE profiles (A and D) and UPGMA tree of 16 S rRNA gene fingerprints (B and E) of the sludge (S1) and soil (S2) enrichment cultures. Samples were taken after 2, 5, 7 and 10 enrichments for the free suspension (FS), gravel (GR) and chips (CH) enrichments. Also Pareto-Lorenz curves based on PCR DGGE analysis of the final enrichments were calculated (C and F)

Table 2-1: Biodiversity of chloropropham degrading bacteria in sludge and soil enrichment cultures after 10 medium refreshments based on culturing on chloropropham film plates. Species were isolated from free suspension (FS), gravel (GR) and/or chips (CH) enrichments and identified using 16S rRNA gene sequencing. The match with the closest type strain of the EMBL database is shown in the right column. The number of genotypically identical strains isolated from the free suspension (FS), gravel (GR) and/or chips (CH) enrichments are indicated in the fourth, fifth and sixth column respectively. The number of colonies that were picked up from the chloropropham film plates but showed no ability to degrade chloropropham in liquid medium are also presented.

Species information			Sludge inoculum			Closest type strain		
Isolate	Accession Nr.	Identification	FS	GR	CH	Type strain	Accession Nr.	sim (%)
R-41380	FR682925	<i>Delftia</i> sp.	7	6	-	<i>Delftia tsuruhatensis</i>	AB075017	99,7%
R-41381	FR682926	<i>Pseudomonas</i> sp.	5	1	-	<i>Pseudomonas umsongensis</i>	AF468450	99,2%
R-41382	FR682927	<i>Pseudomonas</i> sp.	-	3	-	<i>Pseudomonas nitroreducens</i>	AM088473	99,9%
R-41383	FR682928	<i>Delftia</i> sp.	3		4	<i>Delftia acidovorans</i>	AB021417	99,3%
R-41384	FR682929	<i>Achromobacter</i> sp.	2	2	2	<i>Achromobacter denitrificans</i>	AJ278451	99,6%
no chloropropham degradation			5	10	5			
			Soil inoculums					
			FS	GR	CH			
R-41385	FR682930	<i>Diaphorobacter</i> sp.	5	-	-	<i>Diaphorobacter nitroreducens</i>	AB064317	99,9%
R-41388	FR682931	<i>Stenotrophomonas</i> sp.	3	-	-	<i>Stenotrophomonas maltophilia</i>	X95923	99,0%
R-41389	FR682932	<i>Pseudomonas</i> sp.	2	1	-	<i>Pseudomonas nitroreducens</i>	AM088473	99,9%
R-41390	FR682933	<i>Pseudomonas</i> sp.	1	2	-	<i>Pseudomonas umsongensis</i>	AF468450	99,4%
R-41391	FR682934	<i>Achromobacter</i> sp.	2	-	-	<i>Achromobacter spanius</i>	AY170848	99,6%
R-41392	FR682935	<i>Delftia</i> sp.	-	8	7	<i>Delftia acidovorans</i>	AB021417	99,3%
R-41393	FR682936	<i>Pseudomonas</i> sp.	-	1	-	<i>Pseudomonas cedrina subsp. cedrina</i>	AF064461	99,2%
R-41394	FR682937	<i>Pseudomonas</i> sp.	-	2	5	<i>Pseudomonas oryzae</i>	D84004	99,5%
R-41395	FR682938	<i>Achromobacter</i> sp.	-	3	-	<i>Achromobacter xylosoxidans</i>	Y14908	99,8%
R-41398	FR682939	<i>Pseudomonas</i> sp.	-	3	-	<i>Pseudomonas plecoglossicida</i>	AB009457	99,8%
no chloropropham degradation			9	10	7			

Chloropropham removal of sludge and soil enrichment cultures

The indigenous microbial communities of initial sludge and soil enrichment cultures were able to completely remove 50 mg/L chloropropham at a degradation rate of 2.31 ± 0.12 mg CIPC \times day⁻¹ \times gVSS⁻¹ and 2.00 ± 0.08 mg CIPC \times day⁻¹ \times gVSS⁻¹ for the sludge and soil cultures respectively. During this removal, only small amounts of 3-CA (sludge: 0.29 ± 0.16 mg/L; soil: 0.46 ± 0.12 mg/L) could temporarily be detected (Table 2-2).

Table 2-2: Removal rate of chloropropham for the sludge and soil enrichments grown as a free suspension (FS), grown on gravel (GR) and grown on chips (CH). The biofilm enrichments were also tested with the biomass removed from its carrier material (flocs) and the biomass pregrown as a free suspension (fs). The removal rate represents the amount of chloropropham that can be completely degraded per day and per gram of biomass. Tests were done in triplicate. (1) significant difference ($p < 0.05$) compared to the FS culture. (2) significant difference ($p < 0.05$) compared to CH culture. (3) significant difference ($p < 0.05$) compared to the GR culture. (4) significant difference ($p < 0.05$) compared to the CH culture.

Experiment type	Removal rate (mg CIPC \times day ⁻¹ \times gVSS ⁻¹)		temporal 3-CA accumulation (mg \times h/L)		max 3CA- concentration (mg/L)	
	Sludge	Soil	Sludge	Soil	Sludge	Soil
FS	1011.1 ± 20.2	1973.9 ± 39.5	17.0 ± 5.4	112.8 ± 6.0	4.3 ± 1.0	13.3 ± 1.4
GR	703.2 ± 14.1 (1)	1029.9 ± 20.6 (1)	38.9 ± 19.5	11.7 ± 2.3 (1)	2.6 ± 0.2	3.1 ± 0.1 (1)
GR-flocs	701.1 ± 14.1	1055.0 ± 21.1	29.4 ± 7.3	14.4 ± 2.5	3.8 ± 0.2	3.6 ± 0.4
GR-fs	1269.2 ± 25.4 (3)	1509.0 ± 30.2 (3)	18.7 ± 10.7	68.7 ± 5.3 (3)	5.3 ± 0.3 (3)	14.1 ± 0.2 (3)
CH	936.4 ± 18.8 (1)	1532.6 ± 30.7 (1)	4.2 ± 0.7 (1)	1.4 ± 1.2 (1)	1.5 ± 0.1 (1)	0.6 ± 0.1 (1)
CH-flocs	945.6 ± 18.9	1488.0 ± 29.8	6.9 ± 1.1 (2)	9.3 ± 1.1 (2)	2.4 ± 0.2 (2)	3.2 ± 0.1 (2)
CH-fs	1083.0 ± 21.7	1681.4 ± 33.7 (4)	21.9 ± 7.3 (4)	37.7 ± 5.4 (4)	4.6 ± 1.1	9.6 ± 0.3 (4)
Original sample	2.31 ± 0.12	2.00 ± 0.08	0.29 ± 0.16	0.46 ± 0.12	N/A	N/A

No chloropropham removal or 3-CA formation was observed with sterile controls. Experiments were set up to investigate the difference in degradation rate between free suspension, gravel and chips enrichment cultures originating from the sludge and soil. All six enrichment cultures showed immediate chloropropham degradation and were able to completely remove chloropropham and its intermediate 3-CA (Figure 2-3). For both sludge

and soil enrichment cultures, the free suspension cultures were able to remove chloropropham faster compared to the biofilm enrichment cultures. Removal rates of the free suspension enrichment cultures compared to the gravel and chips enrichment cultures were 30.1 and 8.4 % higher for the sludge enrichments and 47.8 and 22.3 % higher for the soil enrichments ($p>0.05$). On the other hand, more 3-CA was accumulated by the free suspension cultures. Maximum 3-CA concentrations of the free suspension enrichment cultures compared to the gravel and chips enrichment cultures were 1.6 and 2.8 times higher for the sludge enrichments and 4.4 and 25.4 times higher for the soil enrichments. The 3-CA concentration \times time areas were calculated to quantify the temporal accumulation of the intermediate. The temporal accumulation of 3-CA of the gravel and chips enrichments were not significantly different ($p>0.05$) and 4.1 times lower compared to the temporal 3-CA accumulation of the free suspension for the sludge enrichments and 9.7 and 82.3 times lower for the soil enrichments. Removal rates and 3-CA build up also differed significantly between the used carrier materials.

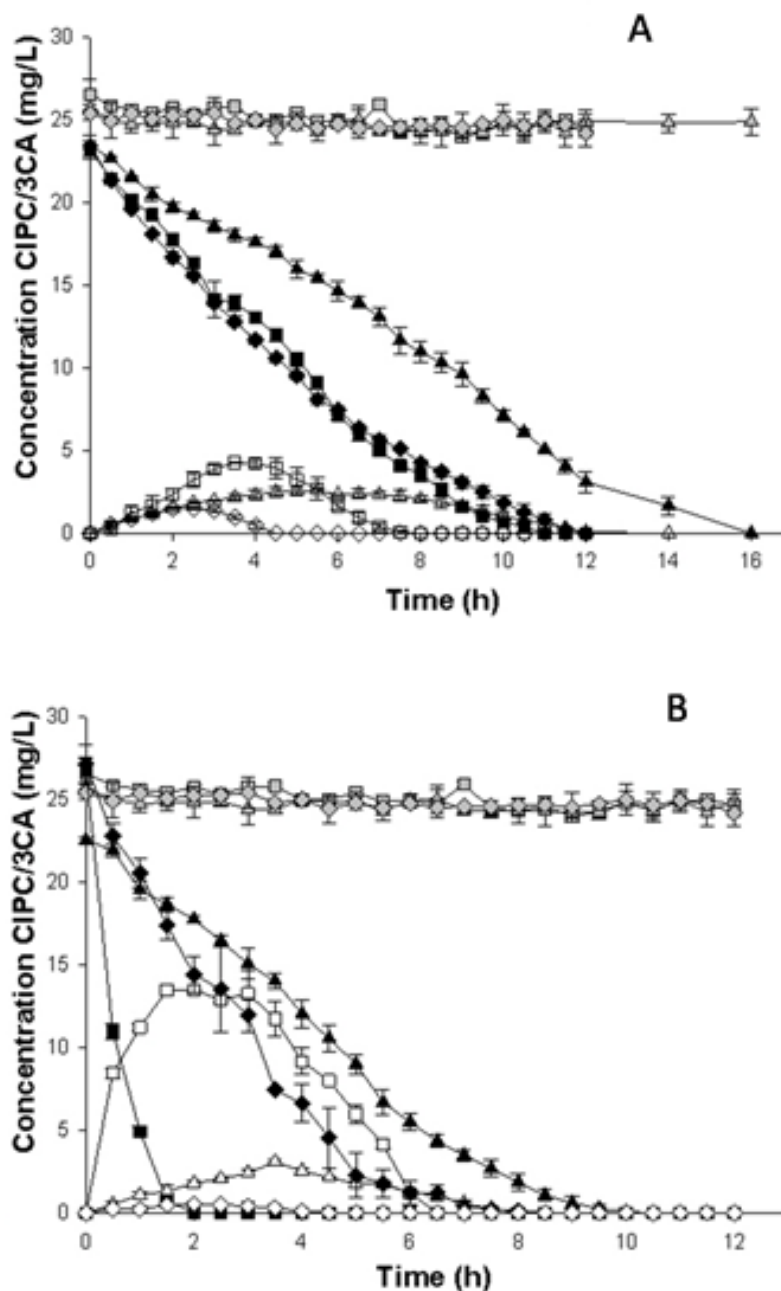


Figure 2-3: Removal of chloropropham (closed symbols) and formation and degradation of 3-chloroaniline (opened symbols) by the sludge (A) and soil (B) enrichment cultures. Free suspension cultures (■, □), gravel biofilm cultures (▲, △) and chips biofilm cultures (◆, ◇) are shown for both sludge and soil enrichments. A combination of heat treated biomass and carrier materials was used as a control for chloropropham removal due to sorption (grey symbols). Data points and error bars represent the means and standard deviations of triplicate measurements. When error bars are not visible, they are hidden behind the symbol. Tests were done in triplicate.

Influence of the biofilm architecture on the chloropropham degradation characteristics

Removing the biofilm biomass from its solid support and subsequently vortexing the culture resulted in the formation of small suspended flocs that were visible under the microscope. Regrowing these flocs in the absence of a solid matrix resulted in the formation of a free suspension culture in which no flocs were microscopically visible. Degradation tests were done for the biofilms attached to their solid support, removed from their solid support (flocs) and regrown as a free suspension (Figure 2-4 A-D). For all biofilm enrichment cultures, there was no significant difference in removal rates of the biofilm fixed to its solid support and the biofilm removed from its solid support ($p > 0.05$) (Table 2-2). The formation of 3-CA in this case was equal for all gravel enrichment cultures but not for the enrichment culture grown on chips. The latter had a higher 3-CA build up which was reflected in an up to six times higher maximum 3-CA concentration and an up to 5.3 times higher temporal 3-CA accumulation for the chips biofilm removed from its solid support compared to the chips biofilm attached to its solid support. Regrowing the biofilm biomass as a free suspension resulted in a chloropropham removal rate that was 8.8 to 44.5 % higher than for the biomass fixed to its solid support. In the case of the sludge chips enrichment, no significant difference was found ($p > 0.05$). Also, more 3-CA was detected. Free suspension biofilm biomass generated 3-CA concentrations that were 2 to 33 times higher compared to biofilm biomass fixed to a solid support. Again when the sludge chips enrichment was tested, no significant difference could be found ($p > 0.05$). Temporal accumulation of 3-CA for the biomass regrown as a free suspension, were between 1.6 and 27.5 times higher compared to the biomass fixed to its solid support for all tested culture types. On average, all chloride mass-balances closed for 96.2 ± 5.7 %. To ensure these differences could not be attributed to changes in the microbial community, DGGE patterns of the free suspension biofilm cultures were compared with the original biofilm cultures. For all cultures between 87.2 and 96.5 % similarity was observed.

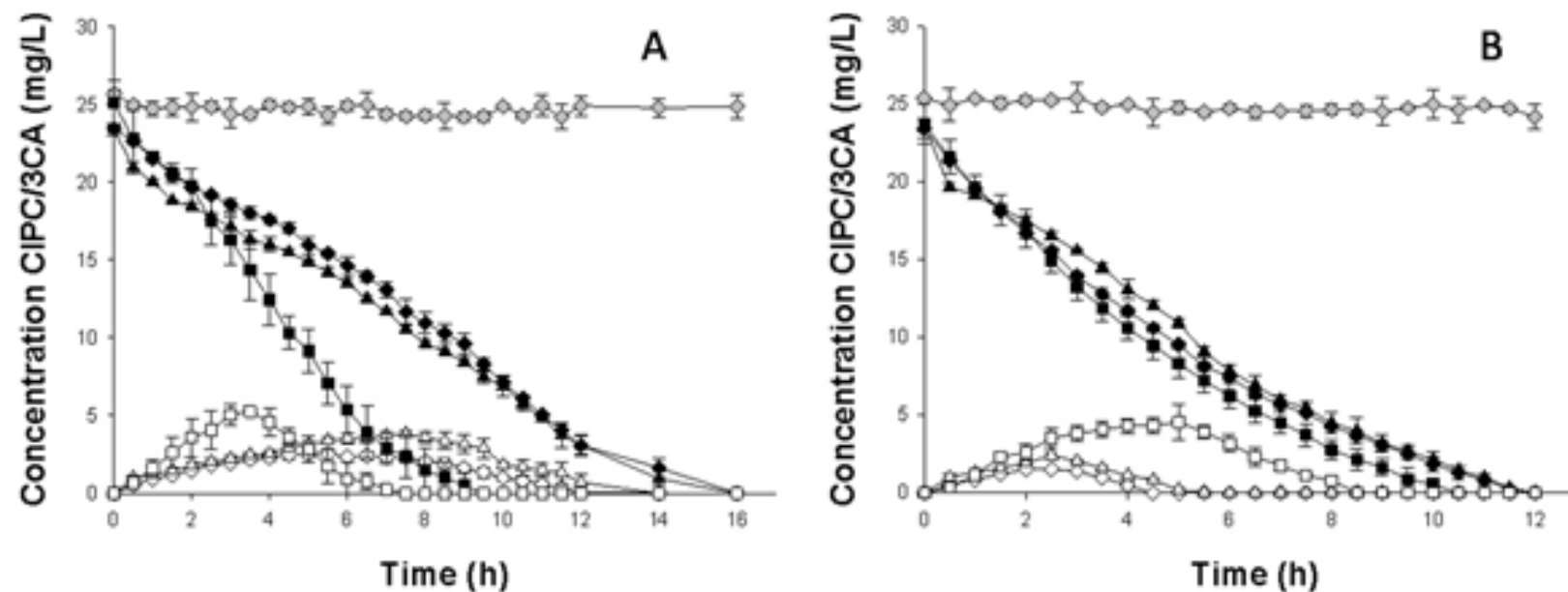


Figure 2-4 (part 1): Removal of chloropropham (closed symbols) and formation and degradation of 3-chloroaniline (opened symbols) by the sludge enrichment culture grown on gravel (A), the sludge enrichment culture grown on biofilm chips (B), the soil enrichment culture grown on gravel (C) and the soil enrichment culture grown on biofilm chips (D). The biofilm fixed to its solid support (\blacklozenge , \lozenge), the biofilm flocs removed from their solid support (\blacktriangle , \triangle) and the biofilm pregrown as a free suspension (\blacksquare , \square) were tested. Heat treated biomass and the appropriate carrier material were used as a control for chloropropham removal due to sorption (grey symbols). Data points and error bars represent the means and standard deviations of triplicate measurements respectively. When error bars are not visible, they are hidden behind the symbols. The figure was divided into two parts to guarantee good visibility of the data points.

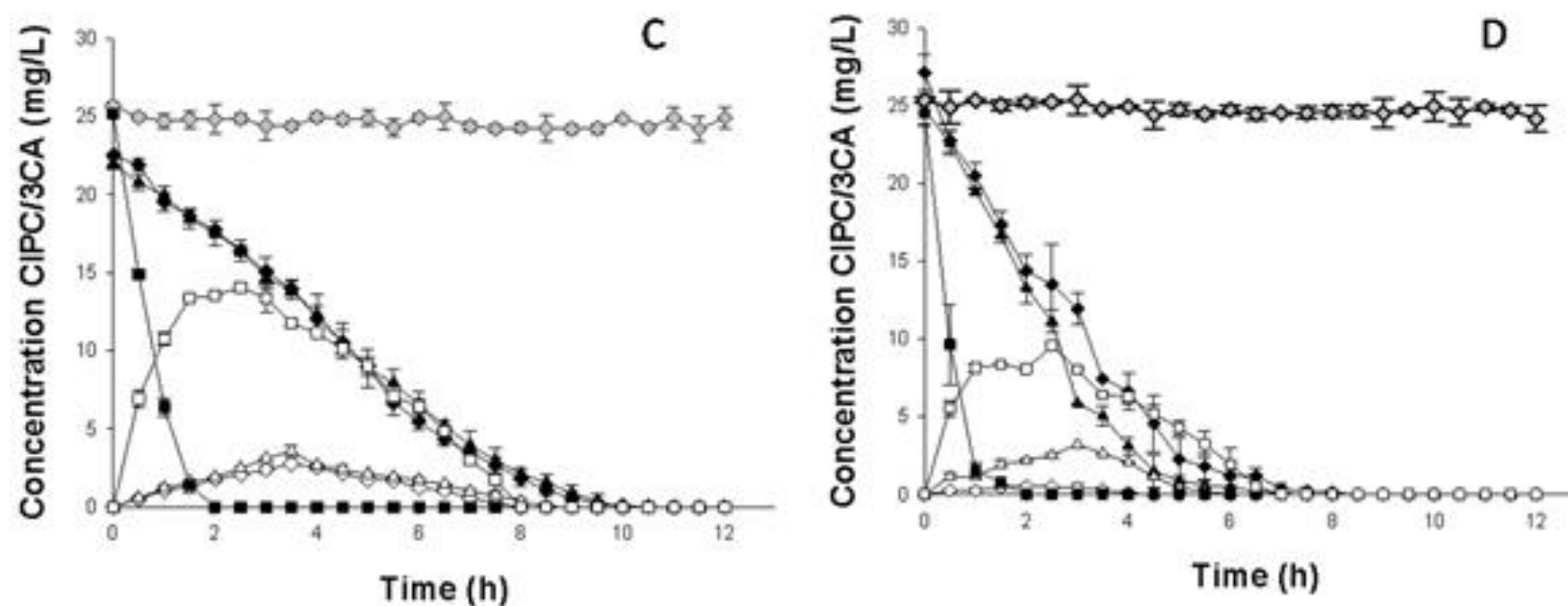


Figure 2-4 (part 2): Removal of chloropropham (closed symbols) and formation and degradation of 3-chloroaniline (opened symbols) by the sludge enrichment culture grown on gravel (A), the sludge enrichment culture grown on biofilm chips (B), the soil enrichment culture grown on gravel (C) and the soil enrichment culture grown on biofilm chips (D). The biofilm fixed to its solid support (\blacklozenge , \lozenge), the biofilm flocs removed from their solid support (\blacktriangle , \triangle) and the biofilm pregrown as a free suspension (\blacksquare , \square) were tested. Heat treated biomass and the appropriate carrier material were used as a control for chloropropham removal due to sorption (grey symbols). Data points and error bars represent the means and standard deviations of triplicate measurements respectively. When error bars are not visible, they are hidden behind the symbol

4 DISCUSSION

In this study, a biofilm based enrichment technique was used to obtain chloropropham degrading microbial biofilm communities and was compared with the commonly used shaken free suspension method. In this new method, chloropropham degrading communities were enriched from contaminated sludge and soil by successive transfers to fresh medium in the presence of two different types of solid supports i.e. gravel and plastic chips. This resulted in the formation of effective chloropropham degrading biofilms on the added carrier materials while in the free suspension enrichments no biofilm formation was observed. 16S rRNA gene based community fingerprinting revealed a clearly different community structure between the free suspension and biofilm enrichments but also according to the used carrier material. Not only the presence but also the type of substratum had a determining influence on the composition of the microbial community. Dominant bands in the DGGE pattern of the biofilm enrichment cultures probably correspond to bacteria that grow well in the presence of a solid support but are outcompeted when no solid support is present because free suspension enrichment favours bacteria that grow more rapidly in suspension (Breugelmans et al. 2007). Other bacteria that are for instance strongly attached to soil particles and proliferate more slowly in free suspension cultures can be outcompeted using this selection procedure. By adding a solid support to the medium, biofilm associated slow growing species are favoured over freely suspended fast growing cells (Bastiaens et al. 2000).

This was also reflected in the chloropropham degrading bacteria that could be isolated from the different enrichment cultures. Although the same sludge or soil inocula were used, different strains were isolated depending on the used method. Especially the soil enrichment culture illustrates the biases inherent to the used enrichment method. Five out of 10 species present in the original soil inocula could only be enriched and isolated using the biofilm enrichment approach, while 3 of the 10 species could only be isolated when the classic free suspension technique was used. Interestingly, the bacterial species isolated from the biofilm enrichment cultures did not form a biofilm when grown in the presence of a solid support, suggesting that interactions with other bacterial species are needed for biofilm formation (Li et al. 2008). When isolating new pollutant degrading species, the biofilm enrichment strategy should be considered as a complementary technique rather

than a replacement for the free suspension enrichment because both techniques tend to select for different kinds of bacteria. On the other hand, when biofilm formation should be desirable in future applications of the catabolic strains, it is recommended to favour biofilm-forming communities during enrichment employing the strategy described here. Members of the genus *Pseudomonas*, *Agrobacterium*, *Flavobacterium*, *Achromobacter* and *Arthrobacter* are known to be able to degrade chloropropham (Kaufman and Kearney 1965; Vega et al. 1985; Marty et al. 1986; Milhomme et al. 1989). *Diaphorobacter sp.*, *Delftia sp.* and *Stenotrophomonas sp.* were not yet proved to be able to hydrolyse chloropropham although they are known for their chloroaniline degrading capacities (Dejonghe et al. 2002; Radianingtyas et al. 2003; Zhang et al. 2010b).

Enrichment of the sludge and soil inocula in the presence of a solid matrix led to microbial communities with a higher species richness and evenness. This result could be expected as in the natural environment, the majority of bacteria grows as a biofilm in close association with solid surfaces and with each other (Costerton et al. 1995; Wimpenny et al. 2000). Enriching these biofilm associated cultures leads to a more diverse microbial population that can potentially better deal with changing environmental conditions while preserving its functionality (Marzorati et al. 2008).

The presence of a solid matrix during enrichment also seemed to have an influence on the metabolic activity of the obtained enrichment cultures. Degradation experiments showed a slightly slower degradation rate but a smaller build-up of 3-CA in case a biofilm was formed. The nature of the solid surface also seemed to influence degradation characteristics because differences in degradation rate and build up of 3-CA were seen between chips and gravel biofilm cultures. The biofilm could influence the chloropropham degradation rate since the cells are embedded in an EPS matrix. This protective barrier hinders diffusion of chloropropham. Also differences in community composition between the free suspension and biofilm enrichments may explain the lower activity of the biofilm cultures. The presence of a solid surface can positively, negatively or not at all affect substrate utilization rate. Van Loosdrecht et al. (Vanloosdrecht et al. 1990) suggested that the results depend on the nature of the microorganism and the kind and concentration of the substrate. A higher build up of 3-CA in free suspension enrichments was reflected in a higher concentration x time surface area compared to the biofilm enrichments. To

unravel the mechanism underlying this observation, 3-CA build up and chloropropham degradation rate were investigated after removing the biofilm from its solid surface and after regrowing the biofilm as a free suspension. No significant differences in removal rate or 3-CA build up were found when the biofilm was removed from its solid support. However, destruction of the biofilm structure by regrowing the biofilm culture as a free suspension led to higher build-up of 3-CA. To our best knowledge, only few authors have investigated the influence of biofilm formation on the accumulation of metabolites. Tafoya-Garnica et al. (Tafoya-Garnica et al. 2009) observed less formation of metabolites during atrazine degradation when a biofilm reactor was used. Our results suggest that the metabolic characteristics biofilm communities are distinct from their free suspension counterparts. Biofilms form spatially well-organised systems that provide the opportunity for metabolic interactions between species (Davey and O'Toole 2000). Chloropropham is first hydrolyzed to 3-CA and isopropanol by the bacterial species present in the biofilm. Although we showed in this study that some species can completely mineralize chloropropham, others are probably only able to degrade 3-CA or isopropanol while others can only do the first hydrolysis step. The metabolic products can thus be exchanged between the species present in the biofilm. Hence less 3-CA is detected in the medium during degradation. Destruction of this elaborate architecture can disrupt this delicate interspecies cooperation and lead to build up of toxic metabolites. A picture of a biofilm on plastic carrier material can be found in figure 2-5.



Figure 2-5: Picture of a biofilm on plastic carrier material

In conclusion, the present study suggests that the presence of a solid support during enrichment can have a great influence on the microbial community composition as the proliferation of biofilm associated bacteria is favoured during the enrichment process. This resulted in different species to be isolated from the biofilm and free suspension enrichment cultures. Enrichment in the presence of a solid support also affects the metabolic characteristics of the obtained enrichment cultures. Slower degradation but less build up of 3-CA is observed when biofilms are formed. The biofilm architecture promoting cross-feeding between the different bacterial species may hold the key to explaining these observations. Clearly, the interactions between the consortium members are complex and further work is needed to elucidate their interplay.

The influence of the biofilm architecture on the pesticide degradation characteristics also suggests that for bioaugmentation of on-farm biopurification systems such as biofilters, phytobacs or biobeds, the use of biofilm catabolic communities might be a proficient alternative to using planktonic freely suspended cultures (De Wilde et al. 2007). On farm biopurification systems are mostly based on sorption and formation of more mobile intermediates (such as 3-chloroaniline) should be avoided as this could lead to a faster breakthrough of the filter system. Biofilms can also protect bacteria against several environmental stresses and wash out from the reactor ((Flemming 1993), (Singh et al. 2006). These advantages combined with a good removal of the mother compound chloroprotham and formation of less 3-chloroaniline could prolong the lifespan of these systems.

CHAPTER 3

INOCULATION WITH A MIXED DEGRADING CULTURE IMPROVES THE PESTICIDE REMOVAL OF AN ON-FARM BIOPURIFICATION SYSTEM

ABSTRACT

To investigate whether the pesticide removal in on-farm biopurification systems (BPS) filled with two different types of substrata (biomix or plastic carriers) is affected by inoculation with a pesticide degrading strain or mixed culture, lab scale BPS used to treat chloropropham point source contaminations were bioaugmented with either a specialized chloropropham degrading strain or a chloropropham degrading enrichment culture. Application of both inoculum types lead to an accelerated degradation activity in the columns filled with plastic carriers. For both substratum types, inoculation with the mixed culture resulted in a lower breakthrough of the toxic intermediate 3-chlororanol at high hydraulic loads, compared to inoculation with the pure isolate and no inoculation. This study suggests that the use of plastic carrier materials could be a proficient alternative to the use of a conventional biomix as a substratum in on farm biopurification systems and that inoculation with a mixed degrading culture can reduce the leaching of more mobile toxic intermediates.

Chapter redrafted after:

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1 INTRODUCTION

The growing amount of pesticide residues in ground and surface water and their impact on human health, biodiversity and overall environmental quality has been widely recognized (Damalas and Eleftherohorinos 2011). Diffuse contamination of pesticides through

percolation, drainage, runoff and spray drift only account for a minor part, while up to 70 % of surface water contamination can be attributed to direct losses by leakages of the spray equipment, spray leftovers and rinsing water from tank cleaning (De Wilde et al. 2007). To address these direct losses, several physicochemical methods have been proposed to treat point source contaminations such as sorption on activated carbon or other materials (Kyriakopoulos and Doulia 2006), oxidation (Chiron et al. 2000) or incineration. Although very efficient, high costs limit the use of these systems on farms (Rose et al. 2003). Moreover, often complete mineralization is not obtained such that further off site treatment is still required.

On farm biopurification systems (BPS) consist of a solid matrix called a biomix, typically composed of straw, peat, soil or other materials, which are able to sorb pesticides. BPS harbor an enormous microbial metabolic potential to eliminate pesticide waste streams and thus provide a safe and economic alternative for physicochemical treatment (De Wilde et al. 2007). Despite good removal capacity for most pesticides in a BPS, some pesticides are poorly degraded resulting in incomplete mineralization of the compound inside the so called biomix (Fogg et al. 2003; Fogg et al. 2004; De Wilde et al. 2009). To enhance rapid and complete degradation, bioaugmentation of the BPS using specialised pesticide degrading bacterial isolates or consortia can be considered. Although applications are known for other pesticide contaminated matrices such as soil (Mertens et al. 2006) and wastewater treatment systems (Boon et al. 2003), to our knowledge no results have been reported of bioaugmentation of BPS using isolated pesticide degrading bacterial strains or consortia.

The hydraulic and chemical load of a BPS is known to influence the leaching of pesticides through a biofilter. A high flow can decrease retention of the pesticide inside the BPS, which decreases the residence time and hence decreases the exposure time to biodegradation (De Wilde et al. 2010a; b). However, these studies do not take into account the possible formation of toxic, more mobile degradation products. These could still leach out of the BPS if they were unaccounted for in the optimization of the hydraulic load.

Chloroprotham, one of the most commonly used sprouting potato sprouting suppressants, was used as a model component in this study (Chapalamadugu and Chaudhry 1992; Tomlin

2006). This compound is slightly to moderately toxic to mammals, birds and aquatic organisms and is moderately persistent in the environment: soil half-lives of 65 days at 15 °C or 30 days at 29 °C have been reported. During bacterial degradation, the even more toxic and persistent metabolite 3-chloroaniline is formed which can be further mineralised. (Tomlin 2006).

In this work, lab scale biopurification systems were filled with an organic mixture or inert plastic carrier materials. These two reactor types were bioaugmented with a single strain or a bacterial consortium (Verhagen et al. 2011). The different systems were compared to each other and to a non-inoculated control reactor concerning the biodegradation dynamics of the pesticide chloropropham and its metabolite 3-chloroaniline, and their microbial community structure. Also, the application of different flow rates was studied to determine its influence on the leaching of chloropropham and 3-chloroaniline.

2 MATERIAL AND METHODS

BPS degradation experiments

Reactor design

Column microcosms consisted of glass 1L columns that were packed with two different types of packing material. The first type of packing material (biomix) consisted of straw (50% v/v), peat (25 % v/v) and soil (25 % v/v) (De Wilde et al. 2007). Substratum amounts were manually mixed in a bucket for 10 minutes to form a homogenous mixture, and packed into the reactors. Compaction of the matrix was done by placing a weight of 5 kg on top of the matrix for 2 minutes. As a second type of packing material, plastic biocarriers (K1 biocarriers, Anoxkaldnes, Sweden) were used. A working volume of 700 mL of each reactor was filled with the appropriate type of packing material. Chloropropham has a maximum solubility of 89 mg/L, so in this study, the influent, water enriched with nutrients (Stanier et al. 1966, see also chapter 2 for the composition of the minimal incubation medium), was spiked with 40 mg.L⁻¹ chloropropham (technical grade, purity >98 %, Certis Europe, Belgium) and dosed to the reactor at an initial flux of 100 L m⁻³.d⁻¹ using a peristaltic pump (type 205S/CA, Watson Marlow, Zwijnaarde, Belgium). The water percolated gravitationally through the column and was collected at the bottom in a glass

Erlenmeyer to which 0.5 mL of phosphoric acid was added to prevent biological breakdown of any chloropropham that might be present in the columns effluent.

Inoculation of the reactors

Steady state flow conditions were established in all reactors prior to inoculation. Minimal incubation medium (not containing any chloropropham) was added to all reactors until the effluent flow was equal to the influent flow. For each of the two packing materials, three inoculation treatments were carried out in duplicate: (i) no inoculation (control) (ii) inoculation with the chloropropham degrading strain *Delftia acidovorans* (iii) inoculation with a mixed chloropropham degrading enrichment culture of unknown composition obtained by Verhagen et al. (Verhagen et al. 2011). The *Delftia acidovorans* strain had been isolated from the exact same chloropropham degrading enrichment culture used in this study (Verhagen et al. 2011). For each type of inoculum, a biomass amount of 50 mg volatile suspended solids (VSS) per L reactor was added to each column.

Influence of the flux on chloropropham and 3-chloroaniline breakthrough

A flux of $100 \text{ L}\cdot\text{m}^{-3}\cdot\text{d}^{-1}$ was applied to all reactors during a period of 150 days, corresponding to 30 pore volumes in case of the biomix reactors and 115 pore volumes in case of the plastic biocarrier reactors. One pore volume represents the time needed for the volume of water that fills the voids of the column to be eluted (De Wilde et al., 2009). After 150 days, the flux was increased 4 times to 200, 400, 800 and $1600 \text{ L m}^{-3}\text{d}^{-1}$ in case of the biomix reactors and 3 times to 200, 400 and $800 \text{ L m}^{-3}\text{d}^{-1}$ in case of the plastic biocarrier reactors. After each pore volume, the amount of chloropropham and 3-chloroaniline in the effluent of the reactors was determined using HPLC-UV. An overview of the reactor en test set-up is presented in Figure 3-1.

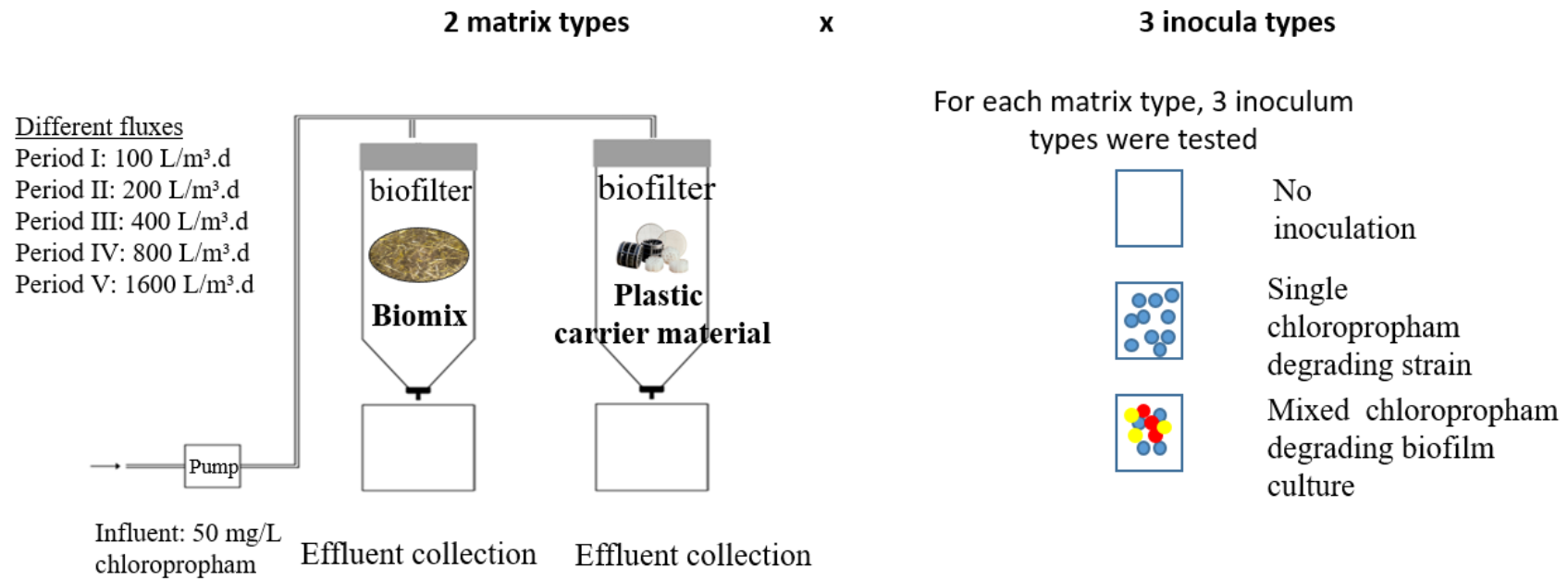


Figure 3-1: Overview of the reactor setup. Two reactors were inoculated for each matrix type and for each inocula type.

Chemical analysis

Supernatants of samples were analysed by reverse-phase HPLC (HP Agilent 1100 series) after cells were removed by centrifugation at 5000 g for 10 minutes. Chloropropham and 3-chloroaniline were analysed using a HPLC system equipped with a G1322A degasser, a G1311A quaternary pump, a G1313A autosampler, a G1314A variable wavelength detector, a G1316A column compartment and HP Chemstation software. A Gracesmart RP-18 column (250- by 4.6-mm inner diameter, 5- μ m particle size; Grace, USA) was used. The mobile phase consisted of CH₃OH/0.1 % H₃PO₄ (60/40) with a flow rate of 1.0 mL min⁻¹ and the UV detector was set to 240 nm. Quantitative determination of chloropropham and 3-chloroaniline was done using an external standard ranging from 0.1 to 60 mg/L. The quantification limit was 0.1 mg.L⁻¹.

Batch degradation experiments

To examine the chloropropham removing efficiency of each column, a batch test was set up in duplicate in 250 mL autoclaved glass erlenmeyers containing 100 mL minimal medium with a final concentration of 25 mg/L chloropropham, 1 g of biomix (taken from the top of the reactor) or 5 plastic biocarriers (also taken from the top of the reactor). Samples were taken at the end of the reactor experiments. To confirm that the removal of chloropropham was biologically driven, a control batch test was set up by adding equal amounts of heat inactivated biomix or biocarriers (20 min, 121 °C) the erlenmeyer flasks. Liquid samples for chloropropham analysis were taken at 30 minutes intervals. This test was performed on day 150 of reactor operation and was done in duplicate for each reactor.

Bacterial community analysis

The template for PCR amplification was obtained by extracting total genomic DNA from the biomass growing on both kinds of packing materials using a procedure described by Boon et al. (Boon et al. 2000). Samples were taken from the top of the appropriate reactor. A 100 μ L aliquot of the crude extract was further purified with the Wizard DNA clean-up kit as described by the manufacturer (Promega, USA). 100 ng of purified genomic DNA was used to amplify 16 S rRNA gene fragments with an Applied Biosystems 2720 Thermocycler using the primers PRBA338fGC and P518r (35 cycles) (Muyzer et al. 1993). Denaturing gradient gel electrophoresis (DGGE) was performed with a denaturing gradient

ranging from 45 to 60 % (Boon et al. 2002). The obtained DGGE patterns were subsequently processed using BioNumerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated based on the Pearson correlation coefficients.

Statistical analysis

Means and standard deviations of steady-state 3-chloroaniline breakthrough amounts for each flux were calculated using the last 6 time points. In case of the batch degradation experiments, 2 degradation tests were performed for each reactor. To test for significant differences between means a one-way ANOVA test was performed using SPSS (IBM SPSS v21). When p-values were lower than 0.05 differences were considered to be significant.

3 RESULTS

Pesticide removal during start-up

For the biomix columns, no chloropropham nor 3-chloroaniline could be detected in the effluent of the inoculated and non inoculated reactors during the first 150 day period. However, the effluent of the reactors with the plastic biocarriers showed considerable concentrations of chloropropham in both inoculated and non-inoculated reactors during this phase of the experiment (Figure 3-2). For the reactors inoculated with the mixed culture and the pure strain, a maximum concentration of $0.8 \pm 0.1 \text{ mg L}^{-1}$ of chloropropham was detected in the different reactor effluents up to 49 and 54 days after inoculation. For the non-inoculated reactors concentrations of chloropropham up to $0.4 \pm 0.1 \text{ mg L}^{-1}$ were detected during the first 67 days. During this whole period, none of the reactors showed any breakthrough of the degradation product 3-chloroaniline. During the first 49 to 67 days, the columns with plastic biocarriers were able to remove at least $98.1 \pm 0.1 \%$ chloropropham from the influent solution. Thereafter the chloropropham removal efficiency in all reactors was 100 % (taking into account the limit of quantification).

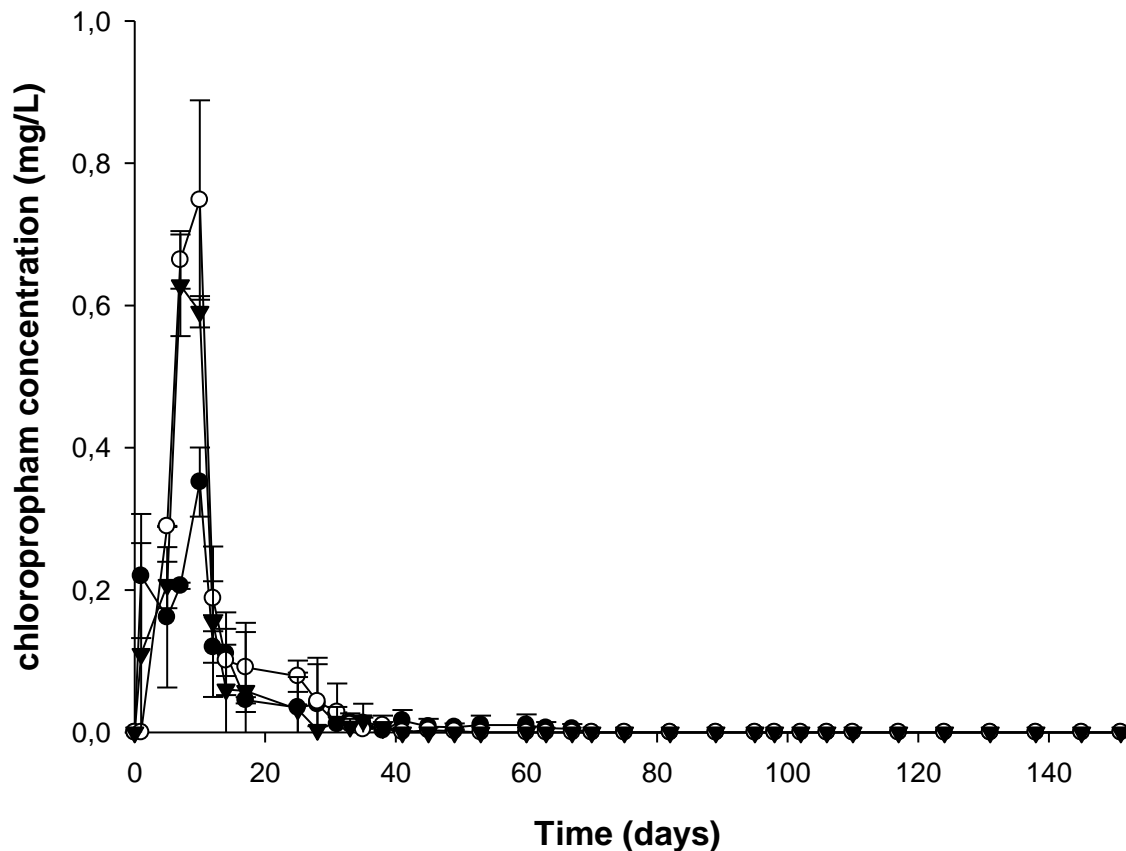


Figure 3-2: Breakthrough of chloropropham in the plastic carrier reactors during the first 150 days of the experiment. Control reactors (●), reactors inoculated with *Delftia acidovorans* (O) and reactors inoculated with a mixed culture (▼) are shown. Experiments were done in duplicate.

Influence of inoculation on the microbial community composition

150 days after inoculation, the cluster analysis of the microbial communities present in the biomix columns (Figure 3-3A) revealed that the control reactors were still very similar ($89 \pm 0\%$) and cluster first with the reactors inoculated with *Delftia acidovorans* displaying at least $72 \pm 3\%$ similarity. Both reactors inoculated with the chloropropham degrading mixed culture form a separate cluster with a similarity of $65 \pm 5\%$ compared to the other four reactors. Concerning the columns filled with plastic biocarriers, cluster analysis (Figure 3-3B) shows a similarity of $75 \pm 0\%$ between the two reactors inoculated with *Delftia acidovorans*, but this cluster is less than $42 \pm 9\%$ similar to all other reactors.

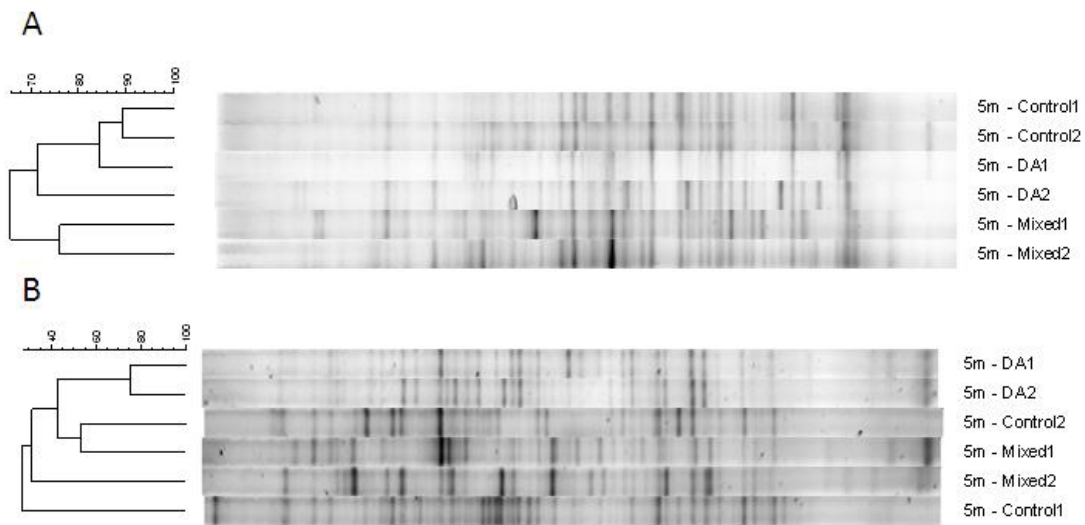


Figure 3-3: DGGE profiles and UPGMA tree of 16 S rRNA gene fingerprints of the microbial communities present in the non-inoculated columns (control1, control2), the columns inoculated with *Delftia acidovorans* (DA1, DA2) and the columns inoculated with the mixed culture (mixed1, mixed2) for both the reactors filled with biomix (A) and the plastic carriers reactors (B). Note different the scales of the similarity-index on the UPGMA trees

Influence of the flux and chloropropham degradation speed

The influence of a higher water flux on chloropropham and 3-chloroaniline breakthrough was investigated. Concentrations are plotted with respect to the amount of pore volume added over the course of time. For the biomix columns, the initial flux of $100 \text{ L/m}^3 \cdot \text{d}^{-1}$ was doubled in four successive periods (Figure 3-4). A flux of $200 \text{ L/m}^3 \cdot \text{d}$, $400 \text{ L/m}^3 \cdot \text{d}^{-1}$ and $800 \text{ L/m}^3 \cdot \text{d}^{-1}$ did not result in a breakthrough of chloropropham nor 3-chloroaniline. When the flux increased to $1600 \text{ L/m}^3 \cdot \text{d}^{-1}$, significant amounts of 3-chloroaniline were found in the effluents of all reactors.

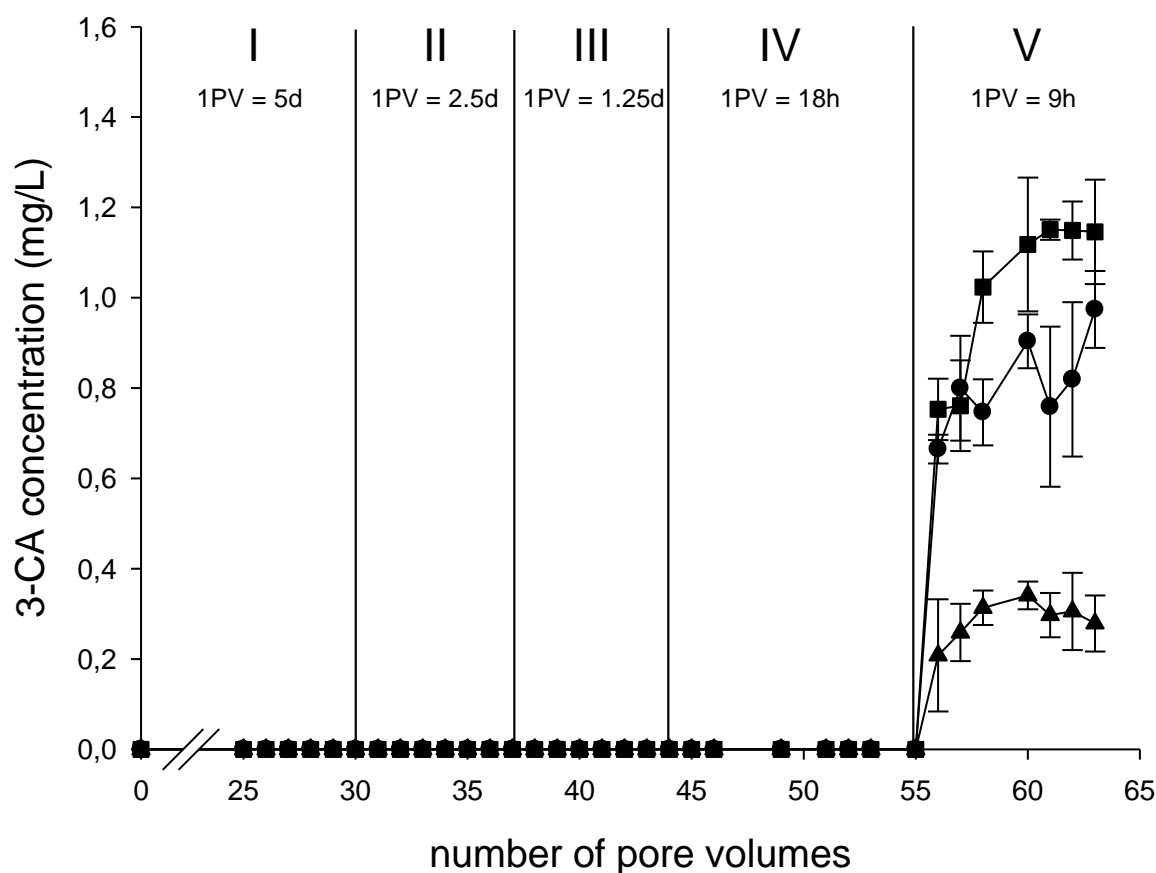


Figure 3-4: Breakthrough of the degradation product 3-CA in the biomix columns. Different fluxes were applied: (I) $100 \text{ L. m}^{-3}.\text{d}^{-1}$ (II) $200 \text{ L. m}^{-3}.\text{d}^{-1}$ (III) $400 \text{ L. m}^{-3}.\text{d}^{-1}$ (IV) $800 \text{ L. m}^{-3}.\text{d}^{-1}$ (V) $1600 \text{ L. m}^{-3}.\text{d}^{-1}$ Non-inoculated columns (●), columns inoculated with the chloropropham degrading species *Delftia acidovorans* (■) and columns inoculated with the chloropropham degrading mixed culture were tested (▲). PV: pore volume. Experiments were done in duplicate.

While the concentration of 3-chloroaniline in the effluent of the reactors inoculated with the *Delftia acidovorans* strain and the non-inoculated reactors did not differ significantly (average concentrations were $1.05 \pm 0.08 \text{ mg.L}^{-1}$ and $0.83 \pm 0.11 \text{ mg.L}^{-1}$ respectively, the reactors inoculated with the mixed culture showed much lower concentrations of 3-chloroaniline. (on average $0.28 \pm 0.06 \text{ mg.L}^{-1}$.) None of the reactors showed any breakthrough of chloropropham.

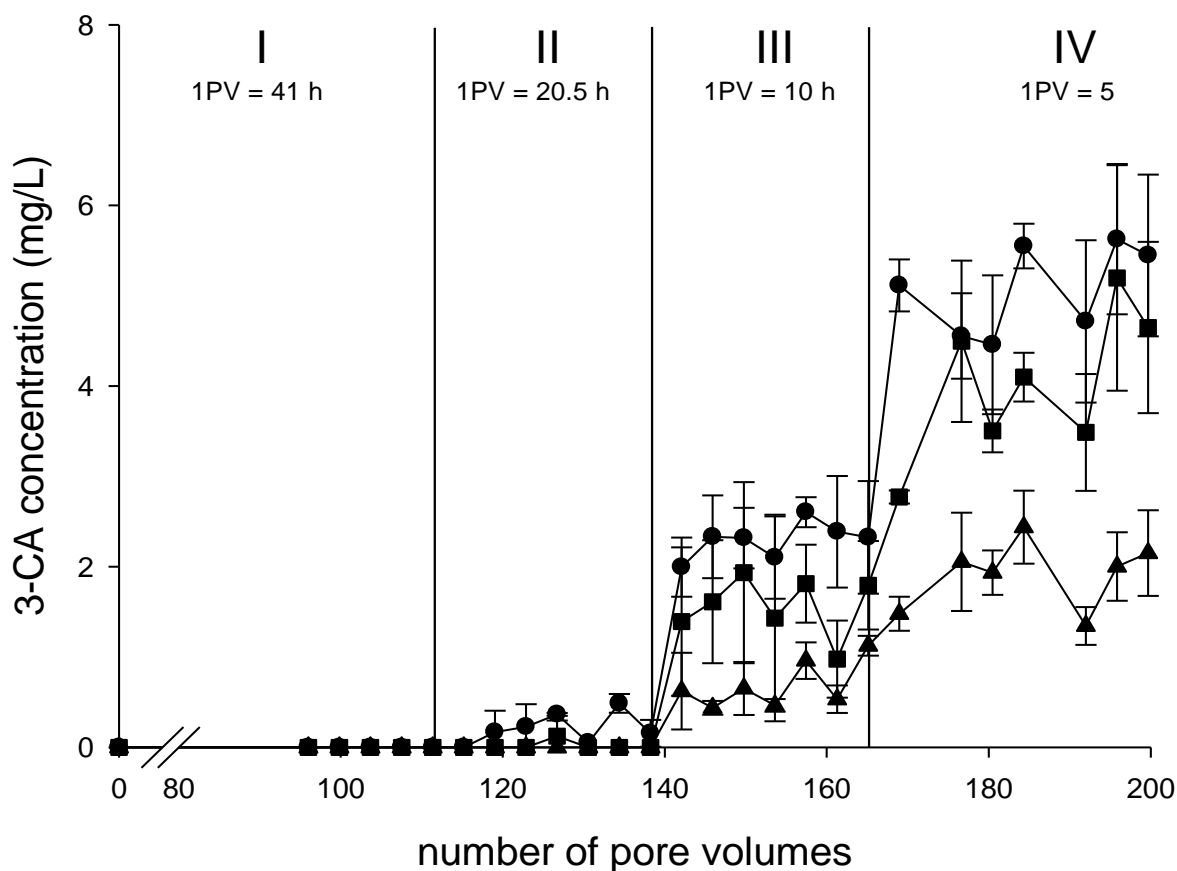


Figure 3-5: Breakthrough of the degradation product 3-CA in the columns filled with plastic carriers. Different fluxes were applied: (I) 100 L. m⁻³.d⁻¹(II) 200 L. m⁻³.d⁻¹(III) 400 L. m⁻³.d⁻¹(IV) 800 L. m⁻³.d⁻¹. Non-inoculated columns (●), columns inoculated with the chloroporpham degrading species *Delftia acidovorans* (■) and columns inoculated with the chloroporpham degrading mixed culture were tested (▲).PV: pore volume. Experiments were done in duplicate.

A similar test was carried out for the columns filled with the plastic biocarriers. The influent flux in these reactors was increased at three points in time (Figure 3-5). A flux of 200 L/ m⁻³.d⁻¹resulted in the breakthrough of very low but detectable amounts of 3-chloroaniline in the columns inoculated with *Delftia acidovorans* and the non-inoculated columns while in the columns with the mixed culture, no 3-chloroaniline was detected. When the flux was increased to 400 L m⁻³.d⁻¹the concentration of 3-chloroaniline found in the columns inoculated with *Delftia acidovorans* and the non-inoculated columns

remained higher than the concentration of 3-chloroaniline found in the columns inoculated with the mixed culture. Doubling the flux one more time resulted in concentrations of 3-chloroaniline in the effluents of the columns inoculated with *Delftia acidovorans* and the non-inoculated columns of $4.0 \pm 0.6 \text{ mg.L}^{-1}$ and $5.1 \pm 0.6 \text{ mg.L}^{-1}$ on average respectively. Also, in these reactor effluents concentrations of chloropropham of up to $1.1 \pm 0.5 \text{ mg.L}^{-1}$ were detected (data not shown). The reactors inoculated with the mixed culture showed significantly lower amounts of 3-chloroaniline in their effluent ($1.9 \pm 0.3 \text{ mg.L}^{-1}$ on average) while no chloropropham was detected ($p < 0.05$).

Batch degradation experiments were carried out after 150 days of reactor operation to establish the chloropropham degrading capabilities of the different microbial communities inside the different reactors (Table 3-1). For the biomix reactors removal rates between 51.5 ± 10.5 and $58.0 \pm 7.5 \text{ mg chloropropham g}^{-1}\text{dw. day}^{-1}$ were observed for the inoculated and non inoculated reactors. However these differences in removal rates were not significant ($p > 0.05$). For the reactors filled with plastic biocarriers, removal rates between 33.7 ± 3.5 and $42.9 \pm 10.2 \text{ mg chloropropham g}^{-1}\text{dw. day}^{-1}$ were observed for the inoculated and non inoculated reactors but these differences were also not significant ($p > 0.05$).

Table 3-1: Removal rate of chloropropham in batch experiments for the biomix and plastic carrier reactors. Non-inoculated columns and columns inoculated with *Delftia acidovorans* or the mixed culture were tested. The removal rate represents the amount of chloropropham that can be completely degraded per day and per gram of biomass. Removal rates cannot be compared between the different substratum materials. Experiments were done in duplicate.

Reactor type	Biomix	Plastic biocarriers
	Removal rate (mg CHLOROPROPHAM g ⁻¹ DWday ⁻¹)	
Control	58.0 ± 7.5	$33,7 \pm 3,5$
<i>Delftia acidovorans</i>	$55,8 \pm 11,3$	$37,2 \pm 7,8$
Mixed culture	$51,5 \pm 10,5$	$42,9 \pm 10,2$

4 DISCUSSION

No detectable concentrations of chloropropham or 3-chloroaniline could be measured in the effluent of any biomix reactor even after 150 days, probably due the combined effect of adsorption and microbial degradation. Both compounds are known for their low mobility in soil (Tomlin 2006) and as substrates for bacterial growth and maintenance (Boon et al. 2000; Verhagen et al. 2011). The biomass present on the biomix collected from all reactors after 150 days was able to completely remove 25 mg.L⁻¹ chloropropham, suggesting a chloropropham and 3-chloroaniline degrading microbial community had developed inside the biomix material of inoculated as well as non-inoculated columns. It has been reported before that repeated pesticide application can lead to the development of an adapted microbial community able to degrade the added pesticides (Arbeli and Fuentes 2007). Tested here as an alternative to biomix substratum, the biocarrier reactors also are to be able to completely remove chloropropham from the influent solution. Although a start-up period is needed before complete removal is obtained, full functionality of the biocarrier BPS can be accelerated through inoculation (49/54 days vs. 67 days). While most on farm biopurification systems use a substratum mix consisted of soil, peat, straw or other organic compounds (De Wilde et al. 2007) our results showed that it may also be possible to use inert carrier materials to host a microbial community able to degrade pesticides. In contrast to decaying organic substrata, inert plastic carrier materials exhibit a very long lifespan, limiting the need to regularly add new substratum material or replace old substratum.

Even after 150 days of operation, the microbial community of the non-inoculated biomix reactors showed a similarity of 89 ± 0 %, demonstrating that the microbial community present in the biomix adapted quite similar to the addition of chloropropham as additional carbon source. This finding implies that reproducibility in key populations is achievable for identical bioreactors. Other authors also described very similar bacterial communities among replicate reactors, where identical selective pressures were applied. (Boon et al. 2003; Lozada et al. 2006; Wittebolle et al. 2009). Adding a chloropropham degrading strain (*Delftia acidovorans*) or a mixed culture resulted in microbial communities that were still 71 ± 2 % and 65 ± 5 % similar to the control reactors respectively, suggesting inoculation has some impact in which the overall microbial community adapted during start-up.

No significant differences in degradation rate during batch experiments could be found between the different inoculation types. In contrast, when a high flux was applied on the reactor columns ($1600 \text{ L.m}^{-3}.\text{d}^{-1}$ in case of the biomix reactors and $800 \text{ L/m}^3.\text{d}$ in case of the biocarrier reactors), differences concerning the breakthrough of chloropropham and 3-chloroaniline could be observed. For both substratum types, the reactors inoculated with the chloropropham degrading mixed culture remove chloropropham and/or its degradation product more efficiently compared to the reactors inoculated with the single *Delftia acidovorans* strain. Another author (Pu and Cutright., 2007) also achieved higher degradation efficiency when a mixed culture was used. He noticed a faster removal of chlorophenol when two types of soil were inoculated with a mixed culture compared to inoculation with two pure cultures. Our results might suggest an overall superior improvement of BPS functionality when bioaugmenting with pesticide degrading enriched mixed cultures.

In on farm applications the average hydraulic load on a biofilter is $20 \text{ L.m}^{-3}.\text{d}^{-1}$. However, in absence of a buffer tank, a biofilter can receive hydraulic loads up to 100 to $200 \text{ L.m}^{-3}.\text{d}^{-1}$ (De Wilde et al. 2009). A biomix biofilter seems to have no problem removing 40 mg.L^{-1} chloropropham under these conditions. In case biocarriers would be used the applied hydraulic load may not exceed $100 \text{ L.m}^{-3}.\text{d}^{-1}$.

In conclusion, it was demonstrated that bioaugmentation with a mixed degrading enrichment culture showed the largest improvement in BPS functionality. Our findings suggest that both plastic carriers and biomix substrata can harbour a stable microbial community that can effectively degrade chloropropham and 3-chloroaniline. The use of plastic carriers is warranted as an alternative non-decaying substratum for BPS as long as the hydraulic load is limited to $100 \text{ L.m}^{-3}.\text{d}^{-1}$ through the use of a buffer tank. The use of these plastic carrier materials could also be used to treat waste streams containing other pesticides such as isoproturon or chlorothalonil which display similar physicochemical and biological characteristics such as adsorption coefficient and half-life (Fogg et al. 2003). However, further research is needed to test the applicability of this method when more mobile and persistent pesticides are used.

CHAPTER 4

SPATIAL HETEROGENEITY IN DEGRADATION CHARACTERISTICS AND MICROBIAL COMMUNITY COMPOSITION OF PESTICIDE BIOPURIFICATION SYSTEMS

ABSTRACT

The aim of this study was to investigate spatial and temporal differences in degradation characteristics and microbial community composition of pesticide biopurification systems. Therefore, pilot scale biofilters were supplemented with the potato sprouting suppressant chloroproham. Two biofilters were inoculated with a chloroproham degrading mixed culture, while the other two were not inoculated. Biodegradation rate, size and composition of the microbial community were monitored during 72 days at different biofilter depths. First of all, results showed that inoculation was not necessary to obtain efficient degradation although it shortens the biofilters start-up period. Secondly, a higher biodegradation rate and chloroproham and 3-chloroaniline degrading microbial community size could be seen in the top part of the inoculated as well as the non inoculated biofilters. Finally, analysis of the microbial community composition shows that no clear spatial stratification of the microbial community could be found in any biofilter. However, analysis of the microbial diversity indicates shows a trend towards increasing microbial diversity in all biofilters and on all biofilter depths, suggesting that during the time of the experiment the biofilters develop a broad carrying capacity in which a genetically very diverse range of chloroproham and 3-chloroaniline degrading species can thrive. A vertical gradient of the chloroproham and 3-chloroaniline degrading community composition, in terms of density and temporal and spatial diversity, was clearly established and was directly connected to a vertical gradient of chloroproham biodegradation activity. The major part of degradation activity takes place in the top part of the biofilter, suggesting that it could be possible to use shorter biofilter reactors or higher loading rates to treat chloroproham waste streams making this type of bioremediation technique economically more feasible.

Chapter redrafted after:

Verhagen, P., Destino, C., Boon, N. and De Gelder, L. (2015) Spatial heterogeneity in degradation characteristics and microbial community composition of pesticide biopurification systems. *Journal of Applied Microbiology* **118**, 368-378.

1 INTRODUCTION

The use of pesticides in agriculture is needed to ensure a stable food production to sustain the growing world population. However, contamination of ground- and surface water caused by excessive use of pesticides may severely menace humans and the ecosystem (Blair and Zahm 1993). Surface water pollutions mainly originate from point source losses which may occur during spills while filling and/or cleaning pesticide tanks or spray material (De Wilde et al. 2007). Installation of an on-farm biopurification system (BPS) has been recommended to treat these point source contaminations. In a BPS the pesticide contaminated waste water from an on-farm cleaning and filling place is collected and fed onto a biologically active solid matrix, called a biomix, which typically consists of straw, soil, peat and other organic materials (Castillo et al. 2008). Inside the BPS the pesticides are removed through sorption and biodegradation processes. Although some BPS achieve high pesticide removal efficiencies, the degradation rate of pesticides and their metabolites that are very persistent or mobile is low (Diez et al. 2013; Urrutia et al. 2013). To enhance rapid and complete degradation, bioaugmentation of the BPS using specialised pesticide degrading bacterial isolates or consortia can be considered (Rahman et al. 2002; Verhagen et al. 2011; Verhagen et al. 2013).

A biofilter is a typical BPS that consists of two or three 1 m³ subsystems depending on the hydraulic load. The different units are connected with each other and stacked in a vertical pile. Pesticide wastewater is brought on top of the first unit and percolates to the other units (De Wilde et al. 2007). Management of this type of BPS requires understanding of pesticide sorption and degradation processes which determine pesticide leaching from the BPS (De Wilde et al. 2007). However, studies on pesticide biodegradation inside a BPS have typically been designed to test the top part of the biomix (Sniegowski et al. 2011b). To our best of knowledge, no information is available on pesticide transformation processes throughout the BPS. In the field, pesticide degradation rates decline with soil depth (Rodriguez-Cruz et al. 2006; Stenrod et al. 2006) although exceptions have been reported (Di et al. 1998; Karpouzias

et al. 2001). It would be interesting to assess whether biodegradation activity varies spatially within a BPS in order to be able to better understand and improve the BPS design parameters.

Although some work has been done investigating the microbial community and activity contributing to the degradation of pesticides inside a BPS (Coppola et al. 2011; Tortella et al. 2013a; Tortella et al. 2013b; Tortella et al. 2013c), information about these phenomena throughout the entire BPS remain to date illusive. However, some work has been done investigating the spatial structure of microbial communities and degradation of volatile organic compounds in biofilters (Tresse et al. 2002; Khammar et al. 2005; Cabrol et al. 2012; Gadal-Mawart et al. 2012). A number of abiotic factors could be important for determining differences in pesticide degradation rate throughout the BPS matrix. Differences in temperature, pH, oxygen concentration and bioavailability of the pesticides can affect microbial growth rates and pesticide degradation (Fomsgaard 1995). The size, richness and composition of the pesticide degrading microbial community at different BPS depths could be important factors ensuring stable pesticide degradation activity.

In this study, chloropropham, one of the most commonly used potato sprouting suppressants, was used as a model compound (Chapalamadugu and Chaudhry 1992). This compound is slightly to moderately toxic to mammals, birds and aquatic organisms and is moderately persistent in the environment. Soil half-lives of 65 days at 15 °C or 30 days at 29 °C have been reported (Chapalamadugu and Chaudhry 1992). Because of its resistance to hydrolysis and oxidation, bacterial degradation is the dominant elimination pathway in the environment (Wolfe et al. 1978). It leads to the formation of the toxic intermediate 3-chloroaniline which can be mineralized further (Kaufman and Kearney 1965).

The current study aimed to answer the following questions (i) Does addition of a chloropropham degrading inoculum decrease the time needed to obtain maximum degradation efficiency? (ii) Does biodegradation rate change with BPS depth and if so, is this reflected in differences in the size of the chloropropham and 3-chloroaniline catabolic communities? (iii) Is there spatial or temporal variability in the composition of the chloropropham and 3-chloroaniline catabolic communities?

2 MATERIAL AND METHODS

Minimal medium

The minimal incubation medium (MM) used in this study was based on Stanier medium (Stanier et al. 1966). It contained in mg L^{-1} 1419.6 Na_2HPO_4 , 1360.9 KH_2PO_4 , 300 $(\text{NH}_4)_2\text{SO}_4$, 98.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.88 $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 2.78 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.69 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.15 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.38 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.24 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.12 $(\text{NH}_4)_6\text{Mo}_{24} \cdot 4\text{H}_2\text{O}$ and 3.2 Na_2EDTA . The liquid minimal medium was supplemented with an appropriate concentration of chloropropham (CIPC, technical grade, purity > 98 %, Certis Europe, Belgium) or 3-chloroaniline (3-CA, 99 %, Alfa Aesar, USA)

Reactor design

The four column microcosms consisted of 1 m high stainless steel reactors with an inner diameter of 9 cm. The columns were filled with a non sterilised packing material consisting of straw (50 % v/v), peat (25 % v/v) and soil (25 % v/v) (de Wilde et al. 2008). To the best of our knowledge, the soil was not previously exposed to chloropropham or 3-chloroaniline. The soil (sandy loam, organic matter content = 5.05 ± 0.24 %, pH = 7.3 ± 0.3) was sieved using a 2 mm sieve and the straw was cut into 5 cm pieces. Substratum amounts were manually mixed in a bucket for 10 minutes to form a homogenous mixture, and packed into the reactors to a density of 0.86 g cm^{-3} . Compaction of the substrate, hereafter called the biomix, was done by placing a weight of 5 kg on top of the biomix for 2 minutes. The used columns could be taken apart at the middle and bottom allowing for biomix samples to be taken on top (0-10 cm), in the middle (50-60 cm) and at the bottom (90-100 cm) of the reactors.

Inoculation of the reactors

During one week, tap water was supplied to the four reactors until steady state flow conditions were established. After that, two replicate reactors were inoculated with a chloropropham degrading mixed culture on top of the reactor and in the middle of the reactor. This culture contained chloropropham degrading strains, amongst which members of the genera *Delftia*, *Achromobacter* and *Pseudomonas*, and was enriched from contaminated soil and described in a previous study by Verhagen et al. (2011). To the top and middle depth of each column, a total inoculum amount of 50 mg volatile suspended solids (VSS) per L reactor was added. Two control reactors without inoculation were set up in parallel. Figure 4-1 gives an overview of the reactor and experimental set-up.

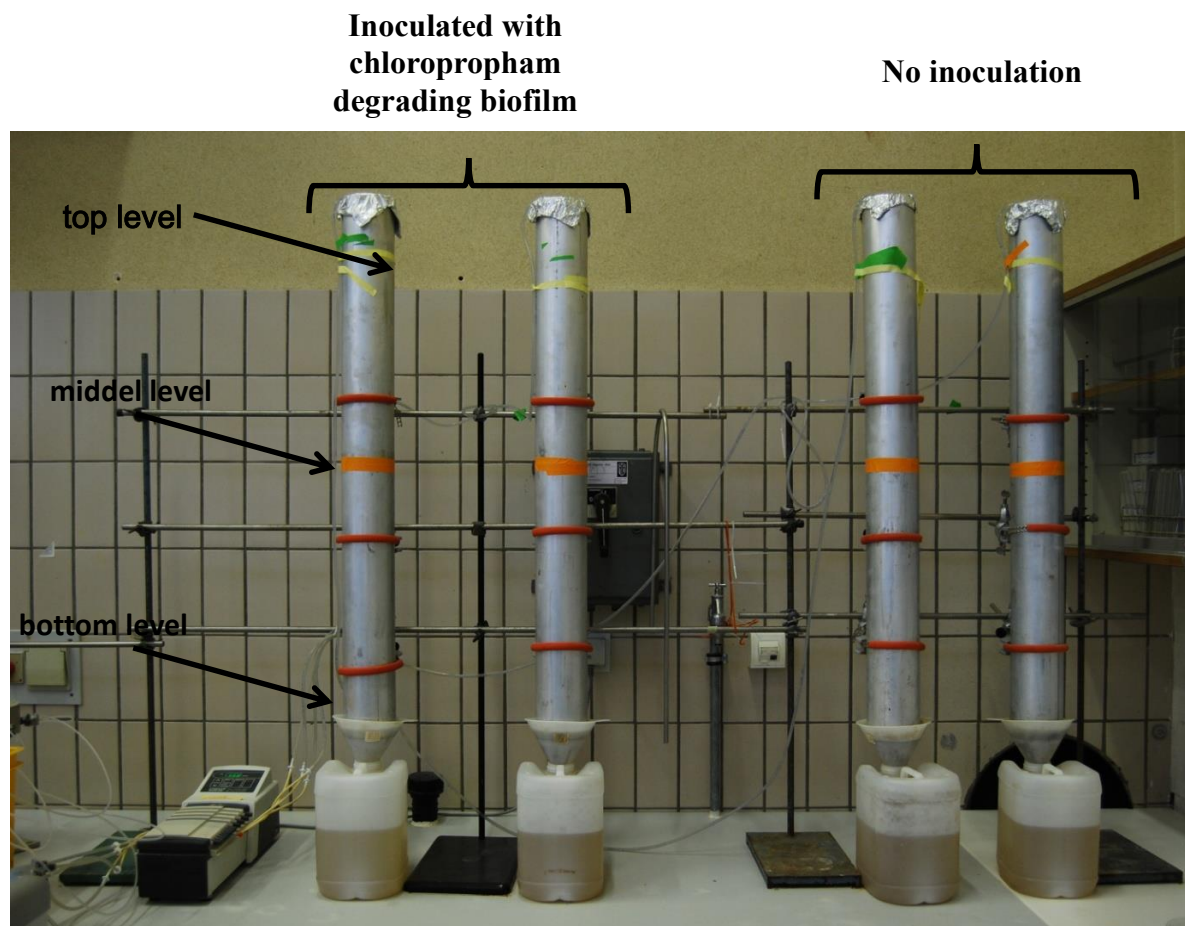


Figure 4-1: Reactor set up. Two reactors were inoculated with a chloropropham degrading biofilm culture. The other two were not inoculated. Samples could be taken at the top level (0-10 cm), the middle level (50-60 cm) and the bottom level (90-100 cm) of the reactors.

Reactor operation parameters

A flux of $100 \text{ L m}^{-3} \text{ d}^{-1}$ was applied to each reactors during a period of 72 days. The added minimal incubation medium was spiked with 50 mg L^{-1} chloropropham (Stanier et al., 1966, see also chapter 2). The reactors were kept at room temperature.

Batch degradation experiments

4, 8, 15, 24, 35, 42, 56 and 72 days after inoculation samples were taken at the top, the middle and the bottom of each column. To examine the chloropropham removal efficiency of the biomass at each depth of the inoculated and non inoculated columns, a batch test was set up in duplicate in 250 mL autoclaved glass Erlenmeyer flasks containing 100 mL minimal medium with a final concentration of 25 mg L^{-1} chloropropham, and 1 g of biomix. To confirm that the removal of chloropropham was biologically driven, a similar batch test was set up by incubating

the erlenmeyer flasks with heat inactivated biomix (20 min, 121 °C). Liquid samples for chloropropham analysis were taken at regular intervals. The degradation rate (amount of CIPC removed per g biomix per day) was calculated. To test for significant differences between means a one-way ANOVA test was performed using SPSS (IBM SPSS v21). When p-values were lower than 0.05 differences were considered to be significant.

Estimation of the abundance of 3-chloroaniline and chloropropham degraders

The Most Probable Number (MPN) method was used. One g of biomix was sampled from each depth of each column at day 4, 8, 15, 24, 35, 42, 56 and 72 and suspended in 50 mL of minimal medium. In a 96 well microtiterplates, 20 μ L of each suspension (only the supernatant was used) was logarithmically diluted up to 10^{-8} in MM (total volume 200 μ l per well) with respectively 200 mg L⁻¹ 3-CA and 89 mg/L CIPC as the selective growth substrate for the two groups. This was done in triplicate for each sample. Non-inoculated wells were used as a control. The CIPC and 3-CA degrader MPN plates were incubated for 2 weeks at 28 °C. Positive wells were scored if growth was detected spectrophotometrically at 620 nm and the abundance of 3-CA and CIPC degraders was calculated using the Most Probable Number method. To test for significant differences between means a one-way ANOVA test was performed using SPSS (IBM SPSS v21). When p-values were lower than 0.05 differences were considered to be significant.

Bacterial community analysis of most abundant degrading strains

In order to capture the microbial diversity of the most dominant 3-CA and CIPC degrading species present in the biomix of each sample, the biomass present from the highest dilution which conferred growth in de MPN assay was transferred to 50 mL of the corresponding medium (MM with 200 mg L⁻¹ 3-CA or 89 mg L⁻¹ CIPC) and incubated for 5 days. The microbial community in this mixed culture therefore consists of the most dominant 3-CA and CIPC degrading species present in the biomix of each sample. The template for PCR amplification was obtained by extracting total genomic DNA from these cultures using the Ultraclean Microbial DNA Isolation Kit (Mobio, USA). 100 ng of purified genomic DNA was used to amplify 16S rRNA gene fragments with an Applied Biosystems 2720 Thermocycler using the primers PRBA338fGC and P518r (35 cycles) (Tresse et al. 2002). Denaturing gradient gel electrophoresis (DGGE) was performed with a denaturing gradient ranging from 45 to 60 % (Tresse et al. 2002). The obtained DGGE patterns were subsequently processed using BioNUMerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). A matrix

of similarities for the densitometric curves of the band patterns was calculated based on the Pearson correlation coefficients. The distribution of the bands in the DGGE pattern can be correlated with the percentage of denaturant gradient of the gel needed to represent the sample's total diversity. The more habitable the environment, the higher the probability it can host a higher number of bands with a wide GC variability (in terms of both percentage and positioning of the GC stretches within the 16S rRNA gene). This concept can be mathematically expressed by the range-weighted richness (Rr) value was calculated based on the total number of bands (N) and the denaturing gradient comprised between the first and the last band of the pattern (Dg), as follows: $Rr = N^2 \times Dg$; The moving window analysis is based on the relative rate of change parameter and averages the degree of change between consecutive DGGE profiles of the same community over a fixed time interval. The matrix of similarities for the densitometric curves of the band patterns was calculated based on the Pearson product moment correlation coefficients and was used to perform cluster analysis. The similarity percentage values were recalculated to change percentage values as follows: % change = 100% - similarity (Marzorati et al. 2008; Marzorati et al. 2013)

Chemical analysis

HPLC analysis

Supernatants of samples were analyzed by reverse-phase high performance liquid chromatography (HPLC) after the cells were removed by centrifugation at 5000 g for 10 minutes. Chloropropham and 3-chloroaniline were analysed using a HPLC system (HP Agilent 1100 series) equipped with a G1322A degasser, a G1311A quaternary pump, a G1313A autosampler, a G1314A variable wavelength detector, a G1316A column compartment and HP Chemstation software. A Gracesmart RP-18 column (250- by 4.6-mm inner diameter, 5- μ m particle size; Grace, USA) was used. The mobile phase consisted of CH₃OH/0.1 % H₃PO₄ (60/40) with a flow rate of 1.0 mL min⁻¹ and the UV detector was set to 240 nm. Quantitative determination of chloropropham and 3-chloroaniline was done using an external standard ranging from 0.1 to 60 mg L⁻¹. The quantification limit was 0.1 mg L⁻¹. The retention time amounted to 11.2 min for chloropropham and 3.4 min for 3-chloroaniline.

Pesticide extraction from the biomix

Extractions of chloropropham and 3-chloroaniline were performed on spare samples of the bottom, middle and top part of the inoculated and non-inoculated columns. Extraction were

carried out on 25.000 ± 0.001 g biomix of which the dry matter content was determined gravimetrically after drying at 105°C during 24h. 100 mL of methanol was added to the biomix and shaken during 1 h at 120 rpm. The liquid phase was separated from the solid phase with a Buchner filter. These steps were repeated three times after which the liquid phase was collected and evaporated. The pesticides were re-dissolved in $\text{CH}_3\text{OH}/0.1\% \text{ H}_3\text{PO}_4$ (60/40) water solution and analysed with HPLC-UV. The average recovery values were $90.8 \pm 0.7\%$ for chloropropham and $91.3 \pm 2.9\%$ for 3-chloroaniline (3 replicates).

3 RESULTS

Effect of inoculation on the time needed to obtain maximum degradation efficiency

Two inoculated biofilters, to which a chloropropham degrading mixed culture was added to the top and middle part of the biomix, and two non-inoculated biofilters were supplied with a chloropropham solution during 72 days. A flux of $100 \text{ L m}^{-3} \text{ d}^{-1}$ did not result in any breakthrough of chloropropham or 3-chloroaniline in the inoculated as well as the non-inoculated reactors (results not shown). To examine the effect of inoculation on the time needed to obtain maximum degradation efficiency, the biodegradation rate of chloropropham was determined after 8, 15, 35, 42, 54 and 72 days at three different depths of the inoculated and non-inoculated reactors (Figure 4-1). In the top part of the inoculated reactors, 8 days after inoculation, the chloropropham degradation rate amounted $76.7 \pm 35.5 \text{ mg CIPC g}^{-1} \text{ day}^{-1}$, which was 8.1 times higher than the degradation rate in the top part of the non-inoculated reactors ($P < 0.05$) (Figure 4-1A). This difference correlated with the amount of chloropropham and 3-chloroaniline degraders that were present in the top part of both reactor types. While the amount of chloropropham and 3-chloroaniline degraders in the top part of the two inoculated reactors at day 8 was $3.1 \pm 0.3 \text{ log MPN g}^{-1}$ and $4.4 \pm 0.3 \text{ log MPN g}^{-1}$ respectively, for the non-inoculated reactors this was below quantification limits (Figure 4-1B). When examining the temporal evolution of the degradation rates in the top part of the inoculated reactors, biodegradation rate was already at maximum 8 days after inoculation, and did not increase significantly over time. However, the number of chloropropham degraders did increase significantly ($P < 0.05$) over time (from $3.1 \pm 0.3 \text{ log MPN g}^{-1}$ at day 8 to $6.4 \pm 0.7 \text{ log MPN g}^{-1}$ at day 72). The number of 3-chloroaniline degrading bacteria evolved in the same way (from 4.4 ± 0.3 to $5.8 \pm 0.2 \text{ log MPN g}^{-1}$) ($P < 0.05$). Figure 4-1 also shows that it takes up to 56 days before the degradation rates in the top part of the inoculated and non-inoculated reactors do not differ significantly anymore.

The biodegradation rate in the middle part of the inoculated reactors was 5.9 times higher than in the non-inoculated reactors. However here it only took 15 days for the differences in biodegradation rate between the middle parts of the inoculated and non-inoculated reactors to become non-significant ($p>0.05$) (Figure 4-1C).

When looking at the bottom part of the reactors, a similar trend was observed (Figure 4-1E). The biodegradation rate in the inoculated reactors is initially 6.2 times higher than in the non-inoculated reactors, while 32 days after inoculation no significant differences in biodegradation rate between inoculated and non-inoculated reactors could be found. In both the middle and bottom part of the inoculated reactors, biodegradation rate was already at maximum 8 days after inoculation and did not increase significantly over time.

Change in biodegradation rate with BPS depth

In all reactors, inoculated and non-inoculated, and for each day, biodegradation rate in the top part of the reactor was significantly higher, while biodegradation rates in the middle and bottom parts of the reactors did not differ significantly. This is also reflected in the amount of chloropropham and 3-chloroaniline degraders found at the different depths of the reactors which was significantly higher ($P<0.05$) in the top part of all reactors while in the bottom and middle part their abundance was the same ($P>0.05$) (Figure 4-1).

Although the same amount of inoculum was added to the middle part of the inoculated reactors, our results show that 8 days after inoculation, biodegradation rate in the top part is 2.4 and 2.6 times higher than in the middle and bottom part. These significant differences are reflected in the amount of chloropropham degraders found in the top part of the inoculated reactors, which on day 8 are 2.1 and 3.1 times higher than in the middle and bottom part respectively ($P<0.05$). Figure 4-1 also shows that 72 days after inoculation, degradation rate in the upper part of the inoculated reactors amounts $124.3 \pm 48.9 \text{ mg CIPC} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$ which is 3.3 and 2.8 times higher than the degradation rate in the middle and bottom part respectively ($P<0.05$). At that day, the amount of chloropropham degraders in the top part of the inoculated reactors is 2.1 and 1.7 times higher than in the middle and bottom parts respectively ($P>0.05$).

Looking at the non-inoculated reactors a similar trend is observed. At day 8, the biodegradation rate amounts $9.4 \pm 1.3 \text{ mg CIPC} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$ in the top part of the reactors while the biodegradation rate in the middle and bottom parts of the reactors were 5.3 ± 0.4 and $4.7 \pm 0.5 \text{ mg CIPC} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$. At day 72, Figure 4-1 shows that the biodegradation rate in the top part of the non

inoculated reactors is 123.9 ± 48.9 mg CIPC $\text{g}^{-1} \text{day}^{-1}$, and 49.1 ± 19.1 and 36.0 ± 7.4 mg CIPC $\text{g}^{-1} \text{day}^{-1}$ in the middle and bottom part of the reactors respectively. Here again we see that the

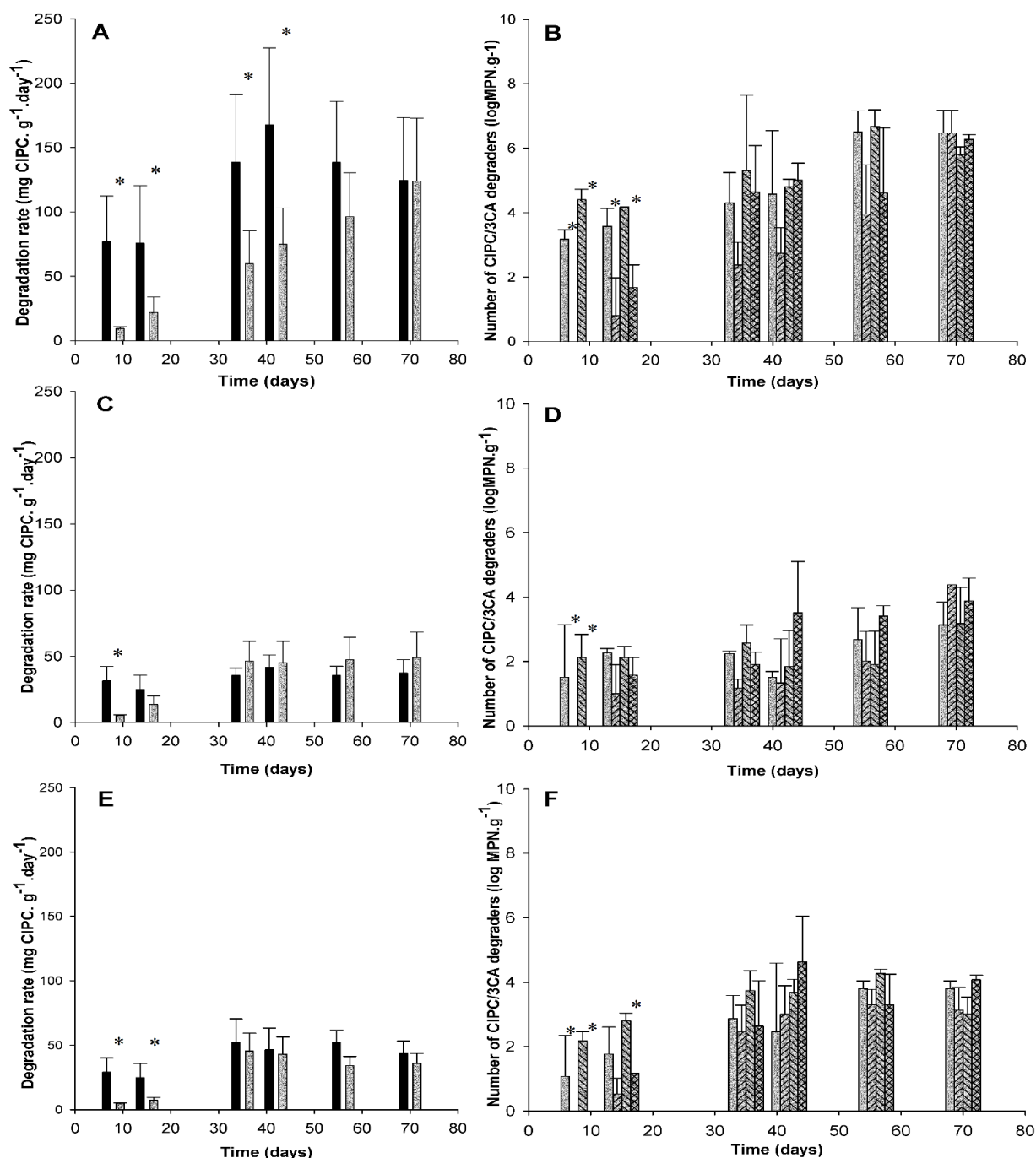


Figure 4-2: Degradation rate in mg CIPC. $\text{g}^{-1}.\text{day}^{-1}$ in the top (A), middle (C) and bottom (E) parts of the inoculated (black) and non inoculated (grey) reactors. Number of chloropropham and 3-chloroaniline degraders in log MPN. g^{-1} in the top (B), middle (D) and bottom (F) parts of the reactors. Number of chloropropham degraders in the inoculated reactors () and the non inoculated reactors (). Number of 3-chloroaniline degraders in the inoculated () and non inoculated reactors(). Experiments were done in duplicate. Results indicated with “*” are statistically different in the inoculated and non inoculated reactors.

number of chloropropham degraders in the reactor top is 1.5 and 2.1 times higher than in the middle and bottom of the reactor respectively. At the end of the experiment, residual amounts of chloropropham and 3-chloroaniline in the top, middle and bottom part of the columns were determined. In none of the columns and at no column depth, chloropropham was found. This means that chloropropham levels were below the detection limit of 0.2 mg kg^{-1} biomix. However, concentrations of 1.9 ± 0.4 and $1.2 \pm 0.2 \text{ mg kg}^{-1}$ of 3-chloroaniline were found in the top part of the inoculated and non inoculated columns respectively. In the middle part respectively $0.6 \pm 0.1 \text{ mg kg}^{-1}$ and $0.8 \pm 0.3 \text{ mg kg}^{-1}$ of 3-chloroaniline was found while in the bottom parts no 3-chloroaniline could be detected. To make sure that oxygen availability did not limit biodegradation rate in any part of the biofilter, oxygen concentration was measured in the top, middle and bottom depths of the columns at the end of the experiment. No concentrations lower than $17.8 \pm 0.6 \%$ (middle) were found. Furthermore, the pH was measured throughout the biomix. No significant differences between depths or treatments were found. An average pH of 7.2 ± 0.3 was measured.

Spatial and temporal variability in the composition of the chloropropham and 3-chloroaniline degrading catabolic communities

The microbial diversity of the chloropropham and 3-chloroaniline degrading community inside the inoculated and non-inoculated reactors is assessed by range weighted richness (Figure 4.2). The microbial diversity of the chloropropham and 3-chloroaniline degrading communities is initially lower at day 15 for the inoculated and non-inoculated biofilters at all depths. However, after 72 days, the microbial diversity of the chloropropham degrading community is between 1.8 and 16.0 times higher at the different depths than at day 15. The microbial diversity of the 3-chloroaniline degrading community follows the same trend: after 72 days, the range weighted richness is between 2.8 and 14.7 times higher than at day 15.

When comparing the microbial diversity of the chloropropham and 3-chloroaniline degrading communities between the different depths, the range weighted richness is 1.2 to 2.1 times lower in top depths of the inoculated and non-inoculated reactors compared to the bottom depths. However only for the 3-chloroaniline degrading community in the inoculated reactor, this difference was found to be significant ($P < 0.05$). At day 72, the range weighted richness of the 3-chloroaniline degrading community is consistently higher than the microbial diversity of the

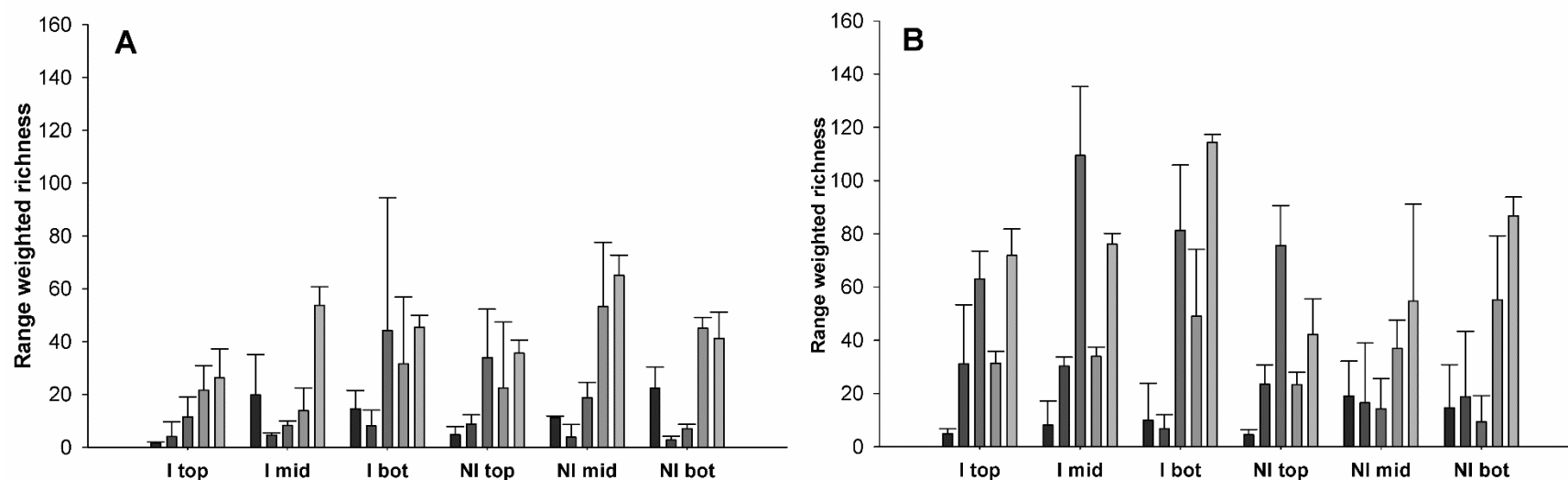


Figure 4-3: Range weighted richness of the chloropropham degrading community (A) and the 3 chloroaniline degrading community (B) of the top, middle and bottom of the inoculated (I) and non inoculated reactors (NI) at day 15 (■), 35 (■), 42 (■), 56 (■) and 72 (■). All values were significantly different between day 15 and day 72 except for the middle of the non inoculated reactors. Experiments were done in duplicate.

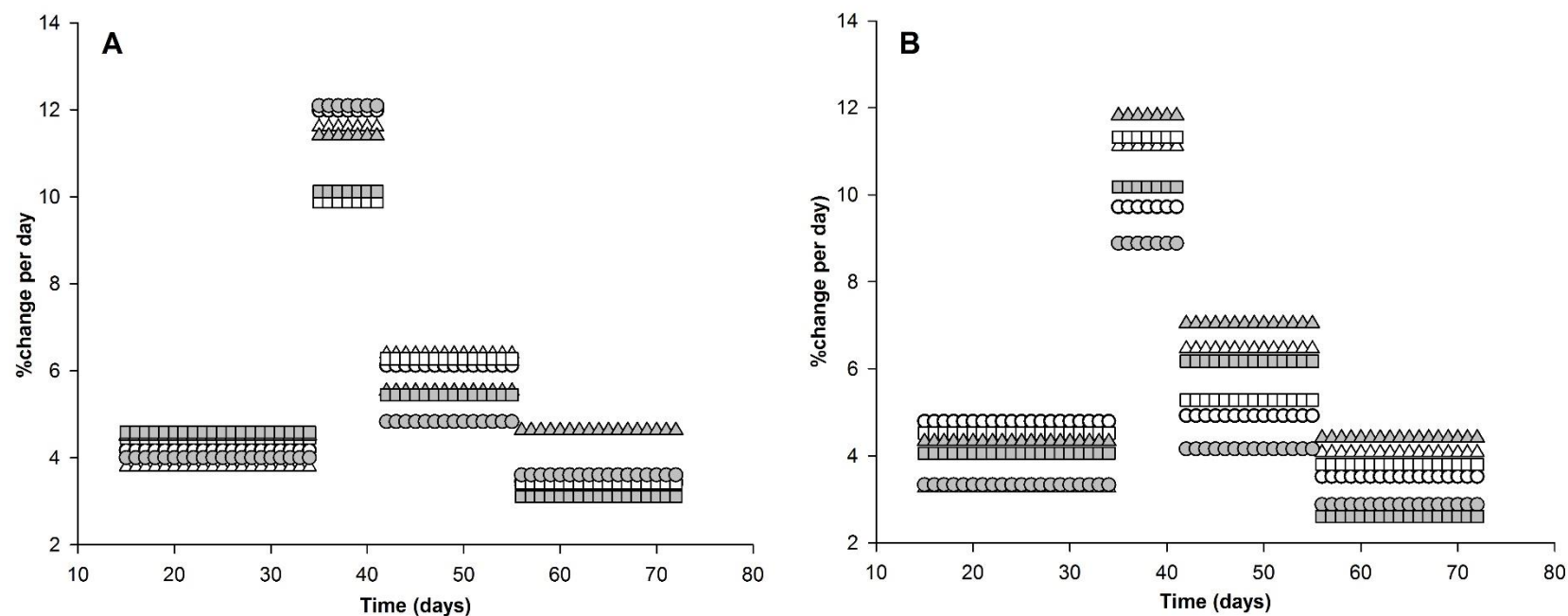


Figure 4-5: Moving window analysis based on the denaturing gradient gel electrophoresis profiles of the chloropropham (A) and 3-chloroaniline (B) degrading microbial communities. Each data point on the graph is the average change per day from day 15 to 35, day 35 to 42, day 42 to 56 and day 56 to 72 for the inoculated (white symbols) and non inoculated reactors (gray symbols) at the top (circle), middle (triangle) and bottom (square) of the reactors.

chloropropham degrading community ($p < 0.05$), except for the middle level of the non inoculated reactors. When comparing the inoculated and non-inoculated reactors, no significant differences could be found in range weighted richness at the different depths after 72 days of reactor operation. This was also reflected in the composition of the microbial diversity after 72 days (Figure 4-3). The cluster analysis shows that the different depths of different reactors do not cluster together. Analysis of the DGGE-patterns with principle component analysis and non metric multidimensional scaling also did not reveal an influence of reactor depth (results not shown). Also, after 72 days, the most dominant chloropropham degrading species that were present in the inoculum are no longer seen in the DGGE patterns of the different biofilter depths.

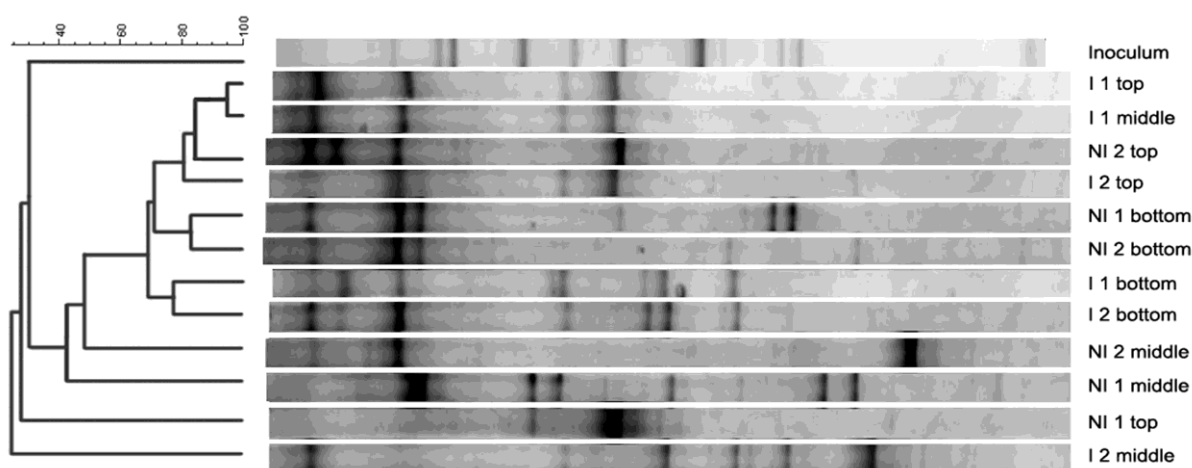


Figure 4-5: DGGE profiles and UPGMA tree of 16S rRNA fingerprints of the most dominant chloropropham degrading bacteria present in the inoculated (I) and non inoculated reactors (NI) for the different depths (top, middle and bottom) 72 days after inoculation.

To evaluate the temporal dynamics of the chloropropham and 3-chloroaniline degrading communities at the different depths of the inoculated and non-inoculated reactors, the change in the microbial community structure within a certain time period was analyzed using moving window analysis (Figure 4-5). From day 15 to day 35, the microbial community of the chloropropham degrading community was quite stable with only a daily change of $4.3 \pm 0.7 \%$ and $4.1 \pm 1.0 \%$ on average for the inoculated and non inoculated reactors respectively. Between day 35 and 42 the chloropropham degrading community had a 2.2 to 3.1 times higher average daily change in microbial community structure compared to the previous period.

Thereafter the microbial community became stable again. The same trend was observed for the 3-chloroaniline degrading community.

4 DISCUSSION

Inoculation with a pesticide degrading culture reduces the amount of time needed to obtain maximum degradation rate

Bioaugmentation is often used as a start-up strategy to establish a pollutant degrading bacterial community inside a reactor (Boon et al. 2002). The improvement of the start-up process is therefore a highly relevant topic in the field of biological wastewater treatment, in particular when the process involves highly persistent pollutants. In this study a chloropropham degrading microbial consortium was used rather than a single strain as it can provide the microbial diversity and robustness needed for ex situ applications (Rahman et al. 2002). In the present study, the inoculated biofilters showed a high removal efficiency from the start of the experiment onwards. However, after 1 week, the non-inoculated reactors were also able to degrade chloropropham and 3-chloroaniline suggesting that the indigenous microbial community inside the biofilter was able to remove chloropropham and 3-chloroaniline although biodegradation rates were not as high as in the inoculated reactors. Due to the retention capacity of the used biomix no breakthrough of chloropropham or 3-chloroaniline was observed for the non inoculated reactors at start-up. The fact that the used biomix has not been in contact with chloropropham or 3-chloroaniline before, suggests that the biomix microbial community may have had an intrinsic capacity to degrade the used pesticide without prior adaptation. It seems that the genes encoding for chloropropham and 3-chloroaniline degradation are quite widespread in the environment.

The set-up of the biofilters, where chloropropham is continuously supplied to the columns, triggers a proliferation of chloropropham and 3-chloroaniline degrading bacteria throughout the entire column. However, the DGGE results after 15 days (results not shown), already show that the original inoculum is no longer visible in the banding patterns of the inoculated reactors suggesting that the bacteria present in the inoculated consortium are no longer dominantly present. This phenomenon is a well-known problem associated with bioaugmentation (Tyagi et al. 2011), for which the maladaptation of the inoculated micro-organisms, competition between introduced and indigenous biomass and predation have been suggested as possible causes of bioaugmentation failure (Goldstein et al. 1985). However, in this study inoculation with a chloropropham degrading mixed culture made it possible to immediately have efficient

degradation in the columns, eliminating the use of a start-up period. This was also seen in a study where addition of terpenes only enhanced biological activity in the biomix during the first incubation days (Tortella et al. 2013c). In the meantime, the indigenous microbial community was allowed to adapt to the added pesticide and become the more dominantly degrading group. This efficient removal of pesticides in the uninoculated columns due to the robust microflora that develops inside the biomixture has also been demonstrated by other authors (Coppola et al. 2011; Tortella et al. 2013a; Tortella et al. 2013b) and can be stimulated by adding inorganic fertilizers (Tortella et al. 2010).

Degradation rate decreases with BPS depth

For the inoculated as well as the non-inoculated biofilters, most biodegradation activity is located at the top where chloropropham and 3-chloroaniline degraders are most abundant and biodegradation rates are the highest. To our best of knowledge, no studies have been done investigating vertical differences in degradation rate in biofilters treating liquid pollutant waste streams. For gaseous waste streams, Khammar et al. (2005) and Gadal-Mawart et al. (2012) did investigate biodegradation efficiencies in biofilters. Due to the environmental conditions in the top layer of these biofilter columns (low humidity and high toxic pollutant concentrations), most compounds were eliminated in the middle and lower parts. In our study, biomix humidity was equal in all parts of the columns while the applied chloropropham and 3-chloroaniline concentrations are not toxic to the microbial community. Moreover, higher availability of chloropropham and 3-chloroaniline in the top part of the reactors resulted in higher degrader density and activity. Also, some studies have been done in the field investigating the relationship between pesticide degradation rate and soil depth. Fomsgaard (1995) and Bending et al. (2006) have also found decreasing degradation rates with increasing soil depth. However, other authors (Karpouzas et al. 2001; de Wilde et al. 2008) have found higher degradation rates in sub-soil compared to top-soil, for which the proposed mechanism entails a decrease in sorption and higher bioavailability of the pesticides linked with a decrease in organic matter content with soil depth. However, as organic matter content is the same in all parts of the biofilters, this mechanism does not apply to our set-up.

Spatial and Temporal variability in the catabolic bacterial community composition

Temporal variability

A lot of bacterial genera such as *Pseudomonas*, *Agrobacterium*, *Flavobacterium*, *Achromobacter*, *Arthrobacter*, *Diaphorobacter*, *Delftia* and *Stenotrophomonas* are already

known for their chloropropham and/or 3-chloroaniline degrading capabilities (Kaufman and Kearney 1965; Vega et al. 1985; Marty et al. 1986; Milhomme et al. 1989; Dejonghe et al. 2002; Radianingtyas et al. 2003; Zhang et al. 2010a; Verhagen et al. 2011). However, the purpose of this experiment was not to identify specific chloropropham or 3-chloroaniline degrading species but to determine the impact of spatial and temporal differences on the microbial community composition in the biofilter columns. To be able to do this, we analyzed the chloropropham and 3-chloroaniline degrading microbial community with DGGE. Although inherent to the used methodology which only cultivable microorganisms were considered, our study allowed to make some remarkable conclusions about temporal and spatial differences in bacterial community composition of these degraders. When looking at temporal differences in the range weighted richness, the microbial diversity of the chloropropham and 3-chloroaniline degrading community was significantly lower in all reactors and on all depths in the beginning of the experiment compared to the end of the experiment (except for the middle of the non inoculated reactors). This means that during the time of the experiment, the environment inside the biofilter probably developed a broad carrying capacity in which a genetically very diverse range of chloropropham and 3-chloroaniline degrading species could thrive (Marzorati et al. 2008).

The moving window analysis also shows that there is a clearly higher change in the microbial community structure between day 35 and 42. During this time period, the range weighted richness also peaks which indicates that a higher number of species than average come to significant dominance. Sometimes higher levels of change in microbial community structure can cause reactor failure because a lot of bacterial species leave the microbial community and come to dominance resulting in overall loss of coherence (Wittebolle et al. 2008). However in this case, a lot of new species are able to enter the microbial community without interacting with the systems functionality i.e. chloropropham degradation. After that, the microbial community becomes less dynamic again, restricting the dominance of new species, guaranteeing the biofilters operational stability.

Spatial variability

Concerning chloropropham degradation rates, there was a clear spatial stratification. Most degradation took place in the top parts of the columns. Although no significant differences ($p < 0.05$) could be established, this was also reflected in a loss of microbial diversity in the top parts of the biofilter columns indicating community specialization. Cabrol et al. (2012)

studying bioremediation of gaseous waste streams using biofilter columns also show spatial stratification in the microbial community structure. However, these differences could not be linked to the systems functionality.

In this study, a vertical gradient of the chloroproham and 3-chloroaniline degrading community composition, in terms of density and temporal and spatial diversity, was clearly established and was directly connected to a vertical gradient of chloroproham biodegradation activity. As the major part of degradation activity takes place in the top part of the biofilters, these results suggest that it could be possible to use shorter biofilter reactors or higher loading rates to treat chloroproham waste streams making this type of bioremediation technique economically even more feasible. Also, inoculation does not seem to be necessary to obtain efficient degradation although it can shorten the biofilters start-up period.

CHAPTER 5

PESTICIDE DEGRADATION USING METHANOTROPHS: INFLUENCE OF PESTICIDE CONCENTRATION AND THE MICROBIAL COMMUNITY COMPOSITION

ABSTRACT

The aims of this study were: (1) To investigate if and at which concentrations pesticides can be co-metabolically degraded by methane oxidizing bacteria (MOB). (2) To investigate the influence of the pesticide concentration on the pesticide removal efficiency of the pesticide (3) To investigate the differences in microbial community composition, pesticide degradation efficiency and maximum inhibition concentration between an sMMO expressing MOB culture and a pMMO expressing MOB culture.

We used an sMMO expressing culture to test the degradation of 14 commonly used pesticides by MOB. 4 pesticides (i.e. chloroprotham, metazachlor ethofumesate and benalaxyl) were successfully removed due to MOB activity. Metazachlor was used to test the influence of the pesticide concentration on the pesticide removal efficiency. This is the amount of pesticide that is removed per amount of methane consumed. Our results showed that a higher pesticide concentration results in a higher pesticide removal efficiency. Furthermore, the pMMO expressing culture also proved to be able to degrade chloroprotham, metazachlor and benalaxyl. However, the degradation efficiency and maximum inhibition concentration for metazachlor was higher for the sMMO expressing culture compared to the pMMO expressing culture. The microbial community analysis showed that type II MOB were dominant in the sMMO expressing culture while type I MOB were dominant in the pMMO expressing culture, suggesting that the two different enzymes were indeed expressed in the two different cultures. As MMO has a much lower substrate specificity compared to heterotrophic bacteria, the usage of methanotrophic bacteria to remediate pesticide contaminated water seems promising.

Chapter redrafted after: Verhagen, P., Benner, J., Claes, L., De Gelder, L. and Boon, N. (submitted) Pesticide degradation using methanotrophs: influence of pesticide concentration and the microbial community

1 INTRODUCTION

Sometimes the microbial capacity to degrade certain pesticides may not be present inside biopurification systems while heterotrophs able to degrade the pesticide may not be available or inoculation is not successful due to biotic or abiotic stress (Gentry et al. 2004). However, some microorganisms are also able to co-metabolize compounds by using non-specific enzymes capable of degrading certain pollutants without the respective microorganisms gaining energy or carbon for microbial growth. The use of methanotrophic bacteria to degrade pollutants could be a viable alternative to the use of pesticide metabolizing bacteria.

Methane oxidizing bacteria (MOB) have the ability to oxidize methane using methane monooxygenase (MMO) as key enzyme. This multicomponent enzyme system which oxygenates methane to methanol, has two distinct forms: soluble MMO which is located in the cytoplasm (sMMO) and membrane associated particulate MMO (pMMO) (Dalton 2005). Although methane is the preferred substrate, both enzymes are known to be able to oxidize other components. sMMO has a broader substrate range compared to pMMO and can oxidize alkanes up to C-8, as well as ethers, cyclic alkanes and aromatic hydrocarbons (Colby et al. 1977; Hou et al. 1979; Burrows et al. 1984). sMMO is also able to oxidize priority pollutants such as halogenated aromatics and biphenyls (Lontoh and Semrau 1998; Han et al. 1999; Lee et al. 2006). pMMO has a narrower substrate range, being able to oxidize alkanes up to C-5 but not being able to oxidize aromatic compounds (Burrows et al. 1984). Although sMMO has been found to have a lower substrate specificity, only few methanotrophs can express sMMO while most can express pMMO (Semrau et al. 2010). Furthermore, genes encoding for sMMO are only expressed when the level of copper is low (Hanson and Hanson 1996; Choi et al. 2003).

Using MOB to enhance pesticide degradation inside on farm biopurification systems seems

a very promising strategy. To the best of our knowledge co-metabolic pesticide degradation using MOB has not yet been reported. This study was set up: (1) To investigate if and at which concentrations pesticides can be co-metabolically degraded by methane oxidizing bacteria (MOB). (2) To investigate the influence of the pesticide concentration on the pesticide removal efficiency of the pesticide (3) To investigate the differences in microbial community composition, pesticide degradation efficiency and maximum inhibition concentration between an sMMO expressing MOB culture and a pMMO expressing MOB culture.

2 MATERIALS AND METHODS

MOB Cultures

The first MOB culture (MOB_{low cu}) was obtained by enrichment of a soil inoculum (Benner *et al.*, 2014) and could grow at low copper concentrations. For this experiment, no copper was added to the nitrate mineral salts (NMS) medium (composition: see Benner *et al.* (2014) to make sure the sMMO enzyme (and not the pMMO enzyme) was expressed.

The second culture (MOB_{high cu}) could grow at high concentrations of copper and was sub-cultivated from the original enrichment culture by van der Ha *et al.* (2013) on NMS medium (with copper). Biomass was sampled from MOB communities growing in active methane oxidizing fed-batch reactors (Kerckhof *et al.* 2014).

DNA extraction and Illumina analysis

DNA-extraction of the samples was done based on the Modified FastDNA® Spin kit (Q-BIO gene). Total DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). The quality of the extracted DNA was evaluated on a 1 % agarose gel. Libraries for the illumina platform (MiSeq) were prepared as previously described by Camirinha-Silva *et al.* (2014) using the primers 807F and 1050R for the V5-V6 region of the 16S ribosomal RNA (rRNA) gene. Definition of operational taxonomic units (OTUs) and data-set quality filters were performed as previously described (Camarinha-Silva *et al.* 2014). The 16S rRNA gene sequence from the closest taxonomic relatives assigned to each of the phylotypes using RDP/NCBI were obtained as a pre-aligned set of manually created sequences from the

SILVA database (Pruesse et al. 2007). A data-set containing the relative abundance of each phylotype in each of the analysed samples was analysed using the software R, version 3.0.2. A table with the abundance of different OTUs and their taxonomic assignments in each sample was generated. Relative abundances were calculated after summing the sequence counts of the OTUs that could be classified on the family level.

Gas composition analysis

Oxygen and methane (~20 % to 0.1 % (v/v)) were measured using a Compact Gas Chromatography (GC) (Global Analyser Solution, The Netherlands) which is equipped with a thermal conductivity detector. For the gas composition measurements, 1 ml of gas sample was taken from the headspace of the bottle using a gas tight syringe (Hamilton, Belgium) and directly injected to the GC.

HPLC analysis

1 mL samples were taken. To conserve the samples, 10 µL of 20 % formic acid was added and the mixture was then filtered with a 0.20 µm syringe filter (Chromafil® PET-20/15 MS). Until analysis, samples were stored at -20°C. Pesticides were analyzed by HPLC consisting of an ACC-300 autosampler, an LPG-3400SD pump and a TCC-3000RS column compartment (Dionex, Sunnyvale, CA, USA) on an Acclaim 120 column (150 mm×4.6 mm, 3 µm). Elution was performed at 25°C and at a flow rate of 0.5 mL min⁻¹. The elution started with 90 % solvent A (0.1 % formic acid) and 10 % solvent B (100 % acetonitrile) for 2 min. Then a linear gradient was used from 90 % to 20 % of solvent A in 35 min, 20 % solvent A during 2 min and from 20 % to 90 % solvent A in 5 min. The injection volume was 20 µL. Detection of the pesticides was performed with a UV–VIS detector. Quantification limits were 0.1 mg/L for imazalil, 0.017 mg/L for metribuzin, 0.017 mg/L for ethofumesate, 0.033 mg/L for pirimicarb, 0.13 mg/L for metsulfuron-methyl, isoproturon, 0.05 mg/L for metazachlor, 0.05 mg/L for benalaxyl, 0.05 mg/L for chloropropham, 0.13 mg/L for terbutylazine, 0.05 mg/L for chloridazon, 0.033 mg/L for chlorotoluron and 0.05 mg/L for carbendazim.

Screening test

To examine which pesticides can be degraded by MOB, 14 pesticides were selected: imazalil, metribuzin, ethofumesate, pirimicarb, metsulfuron-methyl, isoproturon,

metazachlor, benalaxyl, chloropropham, terbutylazine, chloridazaon, chlorotoluron and carbendazim (analytical grade, Dr. Ehrenstorfer, Germany). These compounds are regularly found back in surface water and/or are highly persistent (Peeters et al. 2010; Tomlin 2011). In 125 ml gas tight serum bottles, 30 ml of nitrate mineral salts (NMS) medium was supplemented to the MOB_{low cu} inoculum and 250 µg/L of each pesticide. After addition of 20% CH₄ to the headspace, the bottles were placed on a rotary shaker at 28°C for incubation during a 14 day period. Two treatments were tested (each time in independent triplicates): Treatment A was total degradation at 20 % CH₄ in the headspace, treatment B contained additional 2% acetylene in the headspace as known inhibitor of the methane monooxygenase (MMO) so the MOB were inactive. In this way, heterotrophic removal and removal due to sorption could be evaluated. A growth control containing no pesticides was also incubated to confirm methane removal was not inhibited by the presence of the pesticide. CH₄ and O₂ concentrations were monitored using a compact GC. Whenever CH₄ or O₂ was below 3 %, additional CH₄ or O₂ was added. A sample of the liquid phase was taken at the beginning (day 0) and the end (day 14) of the experiment. Pesticide concentrations were determined using HPLC – UV as described below.

Removal efficiency and maximum inhibition concentration

In 125 ml gas tight serum bottles, 30 ml of nitrate mineral salts (NMS) medium was supplemented to the appropriate MOB inoculum and 500 µg L⁻¹ of chloropropham, benalaxyl or metazachlor. After addition of 20% CH₄ to the headspace, they were placed on a rotary shaker at 28°C for incubation during a period of 14 days. As described above in the screening test, two treatments and a sorption control were tested (each time in independent triplicates). CH₄ and O₂ concentrations were monitored at regular time intervals using a compact GC. Whenever CH₄ or O₂ was below 3 %, additional CH₄ or O₂ was added. A sample of the liquid phase was taken at regular time intervals for analysis with HPLC-UV. A growth control containing no pesticides was also incubated to confirm methane removal was not inhibited by the presence of the pesticide. CH₄ and O₂ concentrations were monitored using a compact GC. An overview of the experimental setup is shown in Figure 5-1. To determine the degradation efficiency the total methane consumption was plotted against the total pesticide removal. Using linear regression analysis (Sigmaplot, USA) a linear correlation between these two variables was tested and

the removal efficiency was determined as the absolute amount of pesticide removed per absolute amount of methane removed. All tests were done in triplicate. The same method was used to determine the maximum inhibition concentration. Solutions with no pesticide added and solutions with 0.25, 0.5, 1.0 and 2.5 mg.L⁻¹ of the selected pesticide were tested. All tests were done in triplicate.

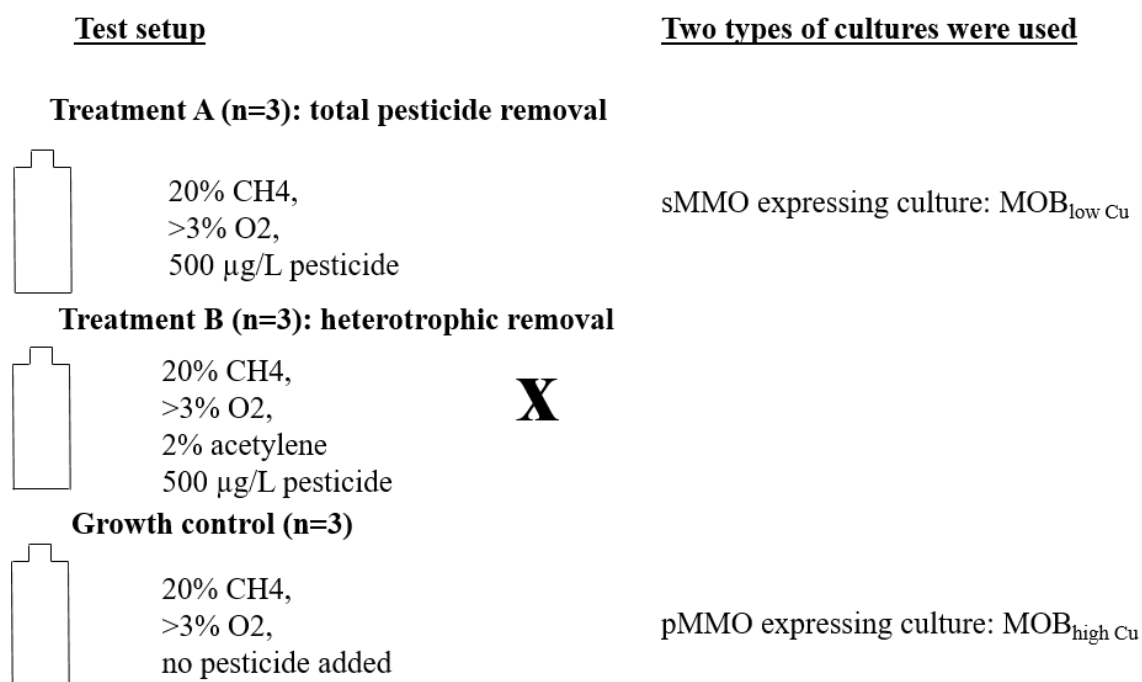


Figure 5-1: Overview of the test setup. Treatment A was used to determine the total pesticide removal (by MOB, heterotrophs, due to sorption). Treatment B was used to determine the total heterotrophic removal or removal due to sorption. A growth control was used to make sure methane removal was not inhibited by pesticide addition. All tests were done in triplicate. Depending on the type of experiment, an sMMO expressing culture (grown in medium with low Cu concentrations) or a pMM expressing culture (grown in medium with high Cu concentrations) was added.

3 RESULTS

Screening for pesticide degradation by methane oxidizing bacteria using the MOB_{lowCu} culture

To assess the degradation potential of MOB for the 14 selected pesticides, a screening test was performed. The MOB_{lowCu} culture was used. No copper was added to the NMS medium so the sMMO enzyme could be expressed. The cultures were incubated during a period of 14 days with 250 $\mu\text{g L}^{-1}$ of the selected pesticide added. Two treatments were tested. Treatment A was total degradation at 20 % CH₄ in the headspace, treatment B contained additional 2% acetylene in the headspace as a known inhibitor of the methane monooxygenase (MMO), so the MOB were inactive. The growth control confirmed that for none of the tests, methane oxidation was inhibited by the presence of the pesticide. Whenever CH₄ or O₂ was below 3 %, additional CH₄ or O₂ was supplemented. After 14 days of incubation, 4 pesticides could be degraded: ethofumesate, metazachlor, benalaxyl and chlorpropham. All other pesticides (imazalil, metribuzin, ethofumesate, pirimicarb, metsulfuron-methyl, isoproturon, terbutylazine, chloridazon, chlorotoluron and carbendazim) were not removed. The treatment tests showed that for none of the removed pesticides, removal could be attributed to sorption or heterotrophic removal. Tests removals up to 11.0 ± 0.9 % for ethofumesate, 70 ± 60 % for benalaxyl, 21 ± 9 % for chlorpropham while metazachlor was removed completely.

Effect of the concentration on the MOB pesticide removal efficiency using the MOB_{low Cu} culture

Using the MOB_{lowCu} culture, cultures were incubated with 250, 500, 1000 and 2500 $\mu\text{g L}^{-1}$ metazachlor (MTZ). Metazachlor and methane removal were monitored (Figure 5-2 A and 5-2 B). The amount of pesticides that was removed was plotted against the amount of methane that was consumed. Using linear regression analysis we showed that for all used concentrations, there was a linear relationship between these two parameters ($p < 0.005$). When the metazachlor concentration is higher, the pesticide removal efficiency is also higher. A metazachlor concentration of 250 $\mu\text{g L}^{-1}$ resulted in a pesticide removal efficiency of 0.08 ± 0.01 $\mu\text{g MTZ}$ per mg methane. When a concentration of 500, 1000 or 2500 $\mu\text{g L}^{-1}$ was used, pesticide removal efficiencies were 0.14 ± 0.02 , 0.32 ± 0.05 and 1.3 ± 0.2 respectively.

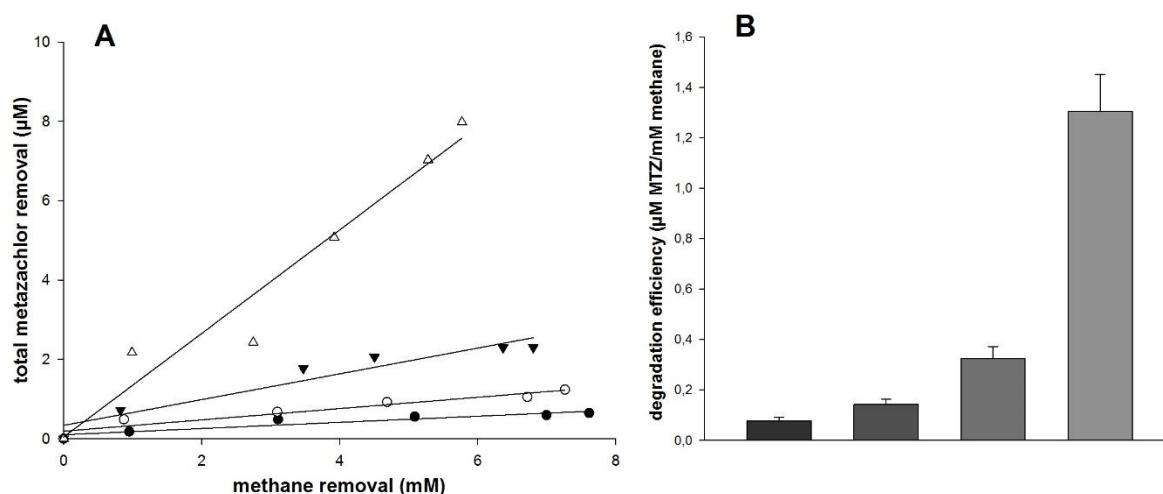


Figure 5-2 A: Total metazachlor removal (µM) vs the amount of methane removed (mM) for different concentrations of metazachlor: 0.25 mg/L (●); 0.5 mg/L (○); 1 mg/L (▼) and 2.5 mg/L (△). The MOB_{lowCu} culture was used. Measurements were done in triplicate.

Figure 5-2 B: Degradation efficiency with standard deviations for different concentrations of metazachlor: 0.25 mg/L (■); 0.5 mg/L (■); 1 mg/L (■) and 2.5 mg/L (■). The degradation efficiency was determined using linear regression analysis. The slope of the best fitting straight line was calculated.

Removal potential of the MOB_{high Cu} culture

To examine if chloropropham, metazachlor and benalaxyl could also be degraded using the MOB_{high Cu} culture, which could grow at high copper concentrations, leading to the expression of the pMMO enzyme, a batch incubation test was set up. The amount of pesticides that was removed was plotted against the amount of methane that was consumed. Using linear regression analysis we showed that for all three pesticides there was a linear relationship between these two parameters ($p < 0.005$). The removal efficiency of metazachlor, chloropropham and benalaxyl amounted 0.021 ± 0.002 , 0.014 ± 0.004 and 0.007 ± 0.001 µM pesticide / mM methane and differed significantly from each other. At the end of the experiment, a total of 35 ± 2 % and 36 ± 7 % of the initial concentration of metazachlor and benalaxyl was removed, respectively. The removal due to MOB activity was determined by calculating the difference between the total removal (treatment A) and the removal due to heterotrophic activity and sorption (treatment B). 80 ± 20 % and 90 ± 40 % of this total removal could be attributed to MOB activity for metazachlor and

benalaxyl respectively (Figure 5-4). For chloropropham, 31 ± 3 % of the initial chloropropham concentration was removed. However, no significant removal due to MOB activity could be found. ($p > 0.05$). To investigate at which concentration of pesticides the methane oxidizing activity of the MOB_{high cu} culture would be inhibited due to the toxic effect of the pesticide, methane oxidizing activity was monitored in medium containing 0, 0.25, 0.5, 0.75, 1 and 2.5 mg/L of the pesticides (Figure 5-3). Our results showed that methane is removed efficiently in the batch tests containing a maximum of 1 mg/L metazachlor, benalaxyl or chloropropham. This means that the maximum inhibition concentration is the same for all tested pesticides.

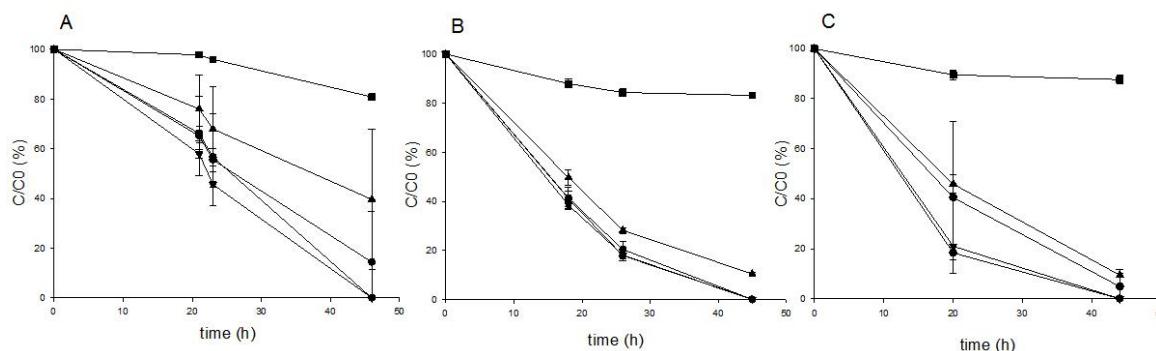


Figure 5-3: Maximum inhibition concentration for the pesticides metazachlor (A), benalaxyl (B) and chloropropham (C). Methane removal percentage C/C_0 as a function of the time for 5 different pesticide concentrations: 0 mg/L (▼), 0.25 mg/L (◆), 0.5 mg/L (●), 1 mg/L (▲) and 2.5 mg/L (■) by the MOB_{high cu} culture. Experiments were done in triplicate.

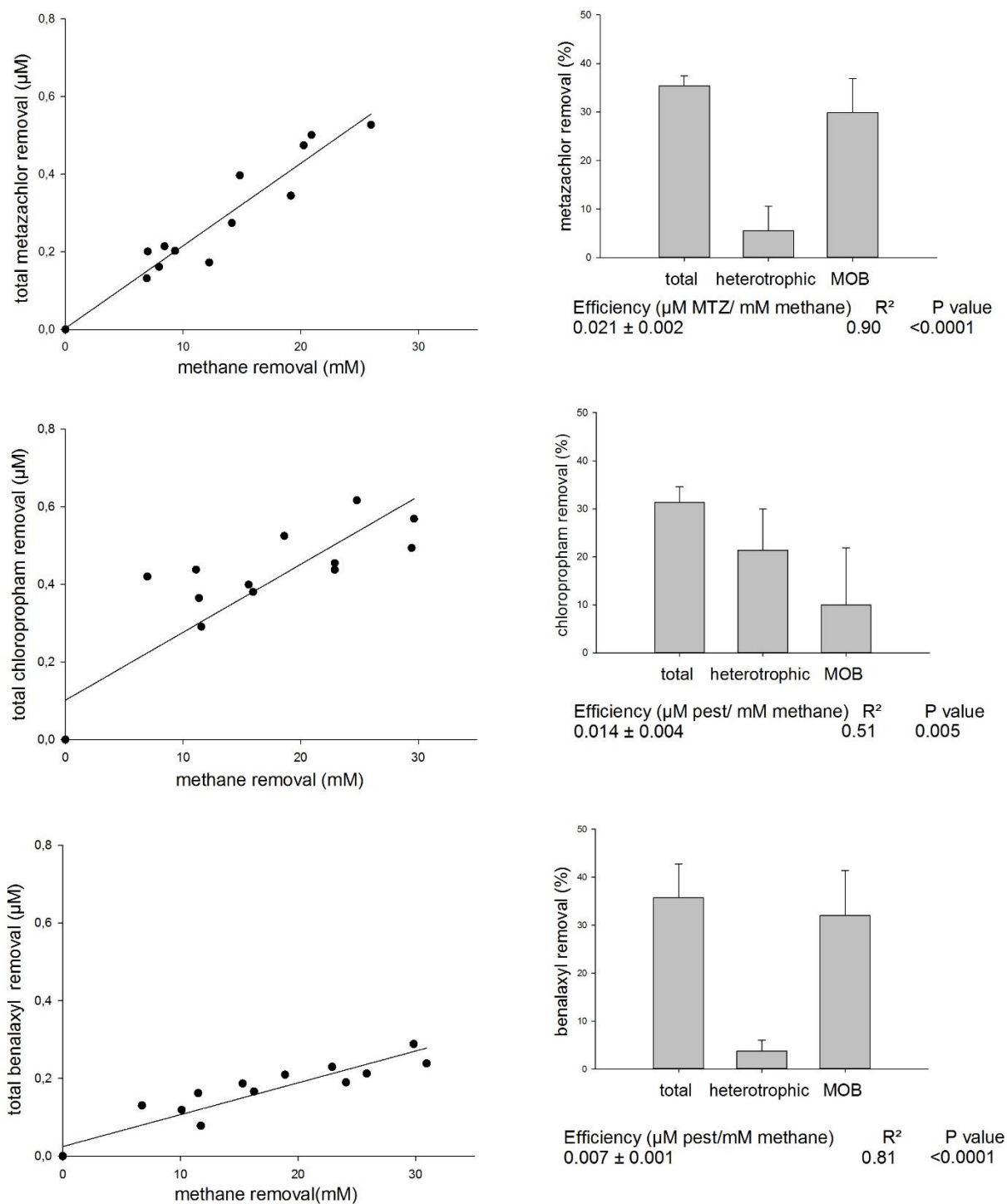


Figure 5-4: Removal efficiency and percentage of removal for 3 different pesticides: metazachlor, benalaxyl and chloroprotham by the MOB_{high cu} culture. The degradation efficiency was determined using linear regression analysis. The slope of the best fitting straight line was calculated using Sigmaplot 10.0. Measurements were done in triplicate.

Effect of the culture type on the metazachlor removal efficiency

Figure 5-2B shows that the metazachlor removal efficiency of the MOB_{low Cu} culture amounts 0.14 ± 0.02 μg MTZ per mg methane (using a 0.5 mg L^{-1} MTZ solution). Figure 5-4 shows that the removal efficiency using the MOB_{high Cu} culture amounted 0.021 ± 0.002 μg MTZ per mg methane. This means that the removal efficiency of the MOB_{low Cu} culture is 7 times higher than the removal efficiency of MOB_{high Cu} culture. There were also differences in maximum inhibition concentration between the MOB_{low Cu} culture and the MOB_{high Cu} culture. The maximum inhibition concentration of the MOB_{low Cu} culture (2.5 mg L^{-1}) was 2.5 times higher than the MIC value of the MOB_{high Cu} culture (1 mg L^{-1}).

Effect of the microbial community composition on the MOB pesticide removal efficiency

The microbial communities of the MOB_{low Cu} culture and MOB_{high Cu} culture were completely different. The main constituents of the MOB_{low Cu} culture belonged to the *Flavobacteriaceae*, *Chitinophagaceae*, *Bradyrhizobiaceae*, *Methylocystaceae*, *Methylophylaceae*, *Moraxellaceae* and *Xanthomonadaceae* families (Figure 5-5 A). The main constituents of the MOB_{high Cu} culture were: *Flavobacteriaceae*, *Chitinophagaceae*, *Methylophylaceae*, *Comamonadaceae* and *Methylococcaceae*. For both cultures; all OTUs classified as methanotrophic *Proteobacteria* were classified into two MOB families: either *Methylococcaceae* (Gammaproteobacteria or type I MOB) or *Methylocystaceae* (Alphaproteobacteria or type II MOB). However, while the *Methylocystaceae* family was an abundant community constituent of the MOB_{low Cu} culture (up to 8.8 % of the total OTU count), it was not dominant in the MOB_{high Cu} culture (0.07 % of the total OTU count). On the other hand, the *Methylococcaceae* family was a very important community constituent of the MOB_{high Cu} culture (25 % of the total OTU count) while it was less important in the soil culture (0.28 % of the total OTU count).

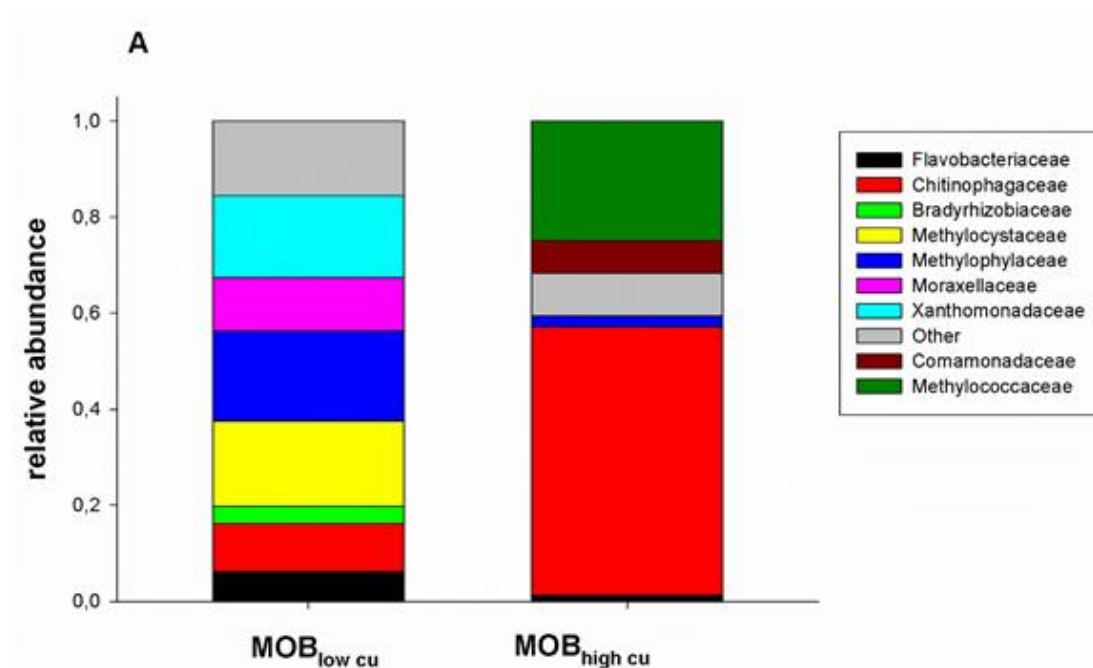


Figure 5-5: Relative abundances of taxa in the MOB_{low Cu} and MOB_{high Cu} cultures. All taxa accounting for more than 1% of the total sequence count are displayed. The RDP classifier, reference set and taxonomy were used. The deepest possible classification is given up to the family level. Relative abundances were calculated after summing the sequence counts of the OTUs that could be classified on the family level.

4 DISCUSSION

Pesticide degradation by methanotrophs

Methanotrophs are known to be able to co-metabolically oxidize pollutants. Depending on the enzyme expressed (pMMO or sMMO) a broad range of components can be degraded (Colby et al. 1977; Hou et al. 1979; Burrows et al. 1984; Lontoh and Semrau 1998; Han et al. 1999; Lee et al. 2006). pMMO has a narrower substrate range and is able to oxidize alkanes up to C-5 but not being able to oxidize aromatic compounds (Burrows et al. 1984). Considering this broader range of substrates oxidized by methanotrophs expressing sMMO, an MOB culture which could grow under sMMO-expressing conditions (i.e. no added copper) was used in our screening tests. We showed that our MOB_{low Cu} culture was able to degrade ethofumesate, benalaxyl, metazachlor and chloropropham. To our best of knowledge, it is the first time that MOB_s have been shown to co-metabolically degrade pesticides. Although a lot of heterotrophs such as *Pseudomonas*, *Agrobacterium*,

Flavobacterium, *Achromobacter*, *Arthrobacter*, *Diaphorobacter*, *Delftia* and *Stenotrophomonas* are able to metabolically degrade chloropropham (Kaufman and Kearney 1965; Vega et al. 1985; Marty et al. 1986; Milhomme et al. 1989; Dejonghe et al. 2002; Radianingtyas et al. 2003; Zhang et al. 2010a; Verhagen et al. 2011), there are no reports of bacterial species being able to metabolically or co-metabolically degrade ethofumesate, benalaxyl or metazachlor. However some authors report the degradation of ethofumesate, benalaxyl and metazachlor in soil (Beulke and Malkomes 2001; Wang et al. 2005; Kucharski and Sadowski 2009; Qin et al. 2014). The degradation test with metazachlor using a methane oxidizing culture at concentrations from 0.25 to 2.5 mg L⁻¹ identified co-metabolism as the main pathway for the degradation of metazachlor. This was supported by the linear relationship between metazachlor removal and CH₄ consumption. A higher pesticide concentration resulted in a higher degradation efficiency: more metazachlor was removed per amount of methane oxidized.

Influence of the culture type

The MOB_{lowCu} was enriched and used in the experiments with a low copper concentration which allows sMMO expression and prevents pMMO expression (no added copper) (Hanson and Hanson 1996; Choi et al. 2003) On the other hand, the MOB_{high cu} culture could oxidize methane at elevated Cu²⁺ concentrations of 10 µmol L⁻¹ which is much higher than the sMMO inhibition concentration of 1 µmol L⁻¹ reported by Begonja and Hrsak (2001) and should eliminate any possible sMMO formation. Although quantitative sMMO analysis based on oxidation of naphthalene was not feasible with our mixed cultures (additional oxidization by heterotrophs cannot be excluded), the Cu²⁺ concentration in the medium allowed us to regulate the expression of sMMO or pMMO.

Under these specific conditions, both cultures were able to co-metabolically degrade metazachlor but at different pesticide degradation efficiencies. The sMMO expressing culture (MOB_{low cu} culture) has a 7 times higher degradation efficiency than the pMMO expressing culture (MOB_{high cu} culture). Likewise, it has been reported that the degradation rate of vinylchloride (VC), trans-dichloroethene (t-DCE) and trichlorethene (TCE) is much faster for sMMO expressing cells of *M. trichosporium* OB3b compared to pMMO expressing cells (Oldenhuis et al. 1991; Lontoh and Semrau 1998; Han et al. 1999; Lee et

al. 2006). Also considering the higher maximum inhibition concentration observed for sMMO expressing cultures (2.5 times higher than pMMO), one might prefer the use of the sMMO expressing cultures over the use of pMMO expressing cultures. On the contrary, Lee et al. (2006) observed faster growth of pMMO expressing cells and faster degradation of VC, t-DCE and TCE when pollutant concentrations were higher than 100 μ M. When pollutant concentrations were lower, sMMO was the preferred enzyme. Furthermore, it should not be forgotten that MOB only catalyze the oxidation step, which leads to the formation of unknown oxidation byproducts which can also have an effect on the maximum inhibition concentration (Oldenhuis et al. 1989; Oldenhuis et al. 1991; Lontoh and Semrau 1998; Han et al. 1999).

The pMMO expressing MOB_{high cu} culture was also able to co-metabolically degrade chloroprotham, benalaxyl and metazachlor. Until now, oxidation of aromatic compounds has only been observed for sMMO. This is the first report of oxidation by pMMO. pMMO shows a different affinity (pesticide removal efficacy) for the different pesticides which has also been reported for other pollutants (Alvarez-Cohen and McCarty 1991). While pMMO is present in almost all MOB, sMMO is mainly limited to type II MOB (Murrell 1992). Both cultures contain representatives of type I (*Methylococcaceae*) as well as type II MOB (*Methylocystaceae*), allowing these cultures to perform in a broader range of circumstances as type I and type II MOB show distinct ecophysiological features (Hanson and Hanson 1996) and have even been suggested to possess different life strategies (Ho et al. 2013). However, our illumina results show that type II MOB (*Methylocystaceae*) are much more dominant in the sMMO expressing soil MOB culture suggesting that sMMO is indeed expressed under the given limited Cu²⁺ concentrations. On the other hand, the pMMO expressing culture contains more type I MOB.

Both cultures contain a lot of heterotrophic bacteria from different families. It has been shown that methanotrophs can support heterotrophic bacteria by supplying a carbon-source and specific nutrients to sustain the growth of the heterotrophic members of a methanotrophic mixed culture (Hrsak and Begonja 2000). However, little is known about the interactions between the methanotrophs and heterotrophs (van der Ha et al. 2013). Nonetheless, these interactions make the community adaptable to various environmental

conditions and more efficient at co-metabolically degrading added components (Hrsak and Begonja 2000; Stock et al. 2013; van der Ha et al. 2013).

In conclusion, this study demonstrates for the first time the successful cometabolic degradation of chloroprotham, metazachlor, benalaxyl and ethofumesate by methane-oxidizing cultures. Both sMMO and pMMO expressing cultures seem to be able to degrade these components although the pesticide degradation efficiency for pMMO degrading cultures is lower at the tested pollutant concentrations. As MMO has a much lower substrate specificity compared to heterotrophic bacteria, the usage of methanotrophic bacteria to remediate contaminated water seems promising.

CHAPTER 6: DISCUSSION, CONCLUSIONS AND PERSPECTIVES

1 POSITIONING OF THIS WORK

Unsatisfactory management of pesticide use in agriculture during the past decades has given rise to harmful concentrations of pesticide residues in surface and groundwater. Most of the surface water contamination by pesticides can be attributed to direct losses such as spillages resulting from filling and leakage of the spray equipment, spray leftovers and technical rest volumes in the tank, pump and brooms (Carter 2000). These point source contaminations, which are mainly confined to the filling and cleaning area of the farm, can be easily collected. Whereas specialized companies can collect and treat these waste streams at high cost to the farmer, it would be beneficial to do so immediately on the farm. Most on-site physico-chemical systems available are very efficient, but in most cases too expensive (Rose et al. 2003). Moreover, complete degradation is often not obtained such that further off-site treatment of waste streams such as sludges is still necessary.

Therefore, biological treatment could be a cheaper, more effective alternative for on-site remediation of pesticide waste streams, making use of microorganisms that ideally completely degrade polluting compounds. Installation of an on-farm biopurification system (BPS) has been recommended to treat these point source contaminations. In a BPS the pesticide contaminated waste water from an on-farm cleaning and filling place is collected and fed onto a biologically active solid matrix, called a biomix, which typically consists of straw, soil, peat and other organic materials (Castillo et al. 2008).

Inside the BPS the pesticides are removed through sorption and biodegradation processes. Pesticide adsorption to the biomix limits the risk for rapid pesticide loss and protects the microbial community from extreme pesticide concentration (Karanasios et al. 2012). Sorption is also a reversible process which allows a sustained bioavailability of the pesticides to the microbial community. Although microbial communities within a BPS might adapt to continuous exposure to pollutants and degrade specific pollutants after a prolonged lag phase, many chemicals or their transformation products remain recalcitrant (von Wiren-Lehr et al. 2001; De Roffignac et al. 2007). A good approach to solve this

problem would be to add the degradation potential needed to remove these recalcitrant pesticides to the system. In this work, metabolic as well as co-metabolic reactions are used to remove pesticides from contaminated wastewater.

Metabolic reactions are growth-linked processes and ideally lead to the complete mineralization of the pollutant. In **Chapter 2**, biofilm cultures were obtained that proved to be able to completely mineralize chloropropham with less formation of the toxic intermediate 3-chloroaniline compared to planctonic cultures. Interestingly, this advantage could be attributed to the biofilm architecture improving nutrient availability and allowing cross-feeding between different species present in the biofilm community. In **Chapter 3** the obtained mixed biofilm cultures as well as pure cultures were used to inoculate pilot scale BPS systems. Inoculation with a mixed degrading culture could reduce the leaching of more mobile toxic intermediates at high hydraulic loads. In **Chapter 4**, inoculation of BPS with a mixed biofilm culture reduced the start-up time needed to obtain efficient degradation while most degradation took part in the top part of the BPS.

Secondly, some microorganisms can also use cometabolic reactions to degrade pollutants. These are reactions that do not sustain the growth of the responsible microorganism and often lead to the formation of transformation products (Benner et al., 2013). In this thesis, methane oxidizing bacteria (MOB), which are known to be able to degrade a wide range of pollutants using their MMO enzyme, are investigated. As MMO has a much lower substrate specificity compared to heterotrophic bacteria, the usage of methanotrophic bacteria to remediate pesticide contaminated water seems promising. In **Chapter 5**, we demonstrated for the first time the successful co-metabolic degradation of 4 pesticides: chloropropham, metazachlor, benalaxyl and ethofumesate. The type of culture (sMMO expressing or pMMO expressing) seemed to be an important factor influencing the pesticide degradation efficiency and the maximum inhibition concentration.

In this chapter we discuss the main outcome of our experiments and translate the acquired results into practical guidelines for the installation and practical use of BPS for maximizing the pesticide degrading efficiency of the system. We also propose directions for future research.

2 DEGRADATION BY BIOFILM CULTURES RESULTS IN LESS FORMATION OF METABOLITES

A lot of research has been done regarding the occurrence of pesticides in ground and surface waters (Holden et al. 1992). However, most studies have focused on the active ingredients (parent compound) without considering transformation products (metabolites) (Kolpin et al. 2000). Complete mineralization of the parent compound rarely occurs in the environment and more persistent, more mobile or more toxic transformation products can be formed (Belfroid et al. 1998). When considering metabolites, most studies focus on a number of dealkylated and hydroxylated metabolites of the chloro-s-triazines, ethanesulfonic and oxanilic acid degradates of chloroacetanilide herbicides such as 2,6-dichlorobenzamide (BAM) from dichlobenyl and aminomethyl phosphoric acid (AMPA) from glyphosate (Scribner et al. 2000; Bjorklund et al. 2011). Other examples of recently discovered metabolites are desphenyl chloridazon and methyl-desphenyl chloridazon of the herbicide chloridazon (Weber et al., 2007; Buttiglieri et al., 2009) and N,N-dimethylsulfamide (DMS) formed from the herbicide chloridazon (Weber et al., 2008). This metabolite has been shown to form the highly toxic N-nitrosodimethylamine upon ozonation during drinking water treatment (Schmidt and Brauch, 2008). Since their discovery, these metabolites are found more regularly and in higher concentrations than their parent compound (Loos et al. 2010a; Loos et al. 2010b). Detection of pesticide metabolites in ground –and surface water is often unexpected and by chance. This has given rise to the question whether some pesticide metabolites of significant importance are still to be discovered (Reemtsma et al. 2013).

Inside a BPS, bacteria can attach to solid particles to form a biofilm. Biofilms form spatially well-organized systems that provide the opportunity for metabolic interactions between species (Davey and O'Toole 2000). In our study, metabolic products such as 3-CA and isopropanol could be exchanged between the species present in the biofilm. Destruction of this elaborate architecture can disrupt this delicate interspecies cooperation and lead to build up of toxic metabolites. The biofilm architecture promoting cross-feeding between the different bacterial species may hold the key to explain these observations. Clearly, the interactions between the consortium members are complex and further work is needed to elucidate their interplay. The influence of the biofilm architecture on the

pesticide degradation characteristics also suggests that for bioaugmentation of on-farm biopurification systems such as biofilters, phytobacs or biobeds, the use of biofilm catabolic communities might be a proficient alternative to using planktonic freely suspended cultures (De Wilde et al. 2007). On farm biopurification systems are mostly based on sorption and formation of more mobile intermediates (such as 3-chloroaniline) should be avoided as this could lead to a faster breakthrough of the filter system. Biofilms can also protect bacteria against several environmental stresses and wash out from the reactor (Flemming 1993; Singh et al. 2006). These advantages combined with a good removal of the mother compound chloropropham and formation of less 3-chloroaniline could prolong the lifespan of these systems.

3 OTHER CARRIER MATERIALS ARE POSSIBLE

Most on farm biopurification systems use a substratum mix consisted of soil, peat, straw or other organic compounds (De Wilde et al., 2007). In contrast to organic substrata, inert plastic carrier materials exhibit a long lifespan, limiting the need to regularly add new substratum materials to replace old substratum. Replacement of substratum materials is expensive as all materials contaminated with pesticides need to be disposed of as hazardous waste. Our findings suggest that both plastic carriers and biomix substrata can harbor a stable microbial community that can effectively degrade chloropropham and 3-chloroaniline. Some questions remain however. First of all, the carrier material needs to be constantly covered with water. This means that water must be uniformly sprayed over the packing material in order to avoid destruction of the biofilm due to drought. Further investigation is needed to assess the effect of extended periods of drought on the operation of the biofilter. Secondly, due to the growth of the biofilm, the packing material may become blocked. This will lead to preferred flows, which will in turn reduce efficiency. If the blockage becomes too severe, the packing will have to be replaced. Replacement of substratum material is expensive as all materials contaminated with pesticides need to be disposed of as hazardous waste. However, during dimensioning it is important to design the biofilter in such a way that the flow is not too high and that growth of the biofilm is controllable in order to avoid blockages. Thirdly, when different pesticides are added, competition between different pesticide degrading communities might lead to reduced

efficiency in the degradation of certain pesticides. Further research is needed to investigate these limitations.

4 CO-METABOLISM IS A PROMISING STRATEGY TO ENHANCE BIODEGRADATION INSIDE A BPS

Some microorganisms can also use cometabolic reactions to degrade pollutants. These are reactions that do not sustain the growth of the responsible microorganism and often lead to the formation of transformation products (Benner et al., 2013). The aim of the last part of this thesis, was to investigate the usage of cometabolic processes to enhance the biodegradation potential of a BPS. The addition of pesticide degrading strains or consortia has been successfully applied and tested in a BPS environment. The use of methane oxidizers in small scale on farm biopurification systems would be a novel strategy. Thanks to their broad oxidizing capabilities, these microorganisms have the potential of carrying out the initial oxidations necessary for the subsequent complete mineralization of these pollutants by heterotrophs (Uchiyama et al. 1992).

Several systems have been proposed for methanotrophic pollutant removal in bioreactors. Yu (2008) identifies two main systems in terms of bioreactor configuration: the single-stage bioreactor and the multi-stage bioreactor. In the single-stage bioreactor, methane degradation (growth) and pollutant removal takes place in the same reactor. Typically, the reactors are seeded with a pure or mixed culture of methanotrophs. Next, methane, air and nutrients are introduced to allow cell growth. Finally, pollutant contaminated water is passed through the reactor. Depending on the way the bacterial cells are retained inside the reactor different single-stage reactor types can be distinguished: expended-bed reactors (Phelps et al. 1990; Phelps et al. 1991), packed-bed biofilm reactors (Strandberg et al. 1989; Speitel and Leonard 1992; Speitel and Segar 1995; Fitch et al. 1996), fluidized bed reactors (Clapp et al. 1999), rotating cylinder biofilm reactors (Arvin 1991) and immobilized soil bioreactors (Karamanev and Samson 1998). Because in these reactors, the growth of methanotrophs and pollutant degradation must occur simultaneously, there is competition between the growth substrate methane and the co-metabolic substrate (pollutant) for the MMO enzyme, resulting in competitive inhibition between the two substrates. Beck (2000) determined the optimal ratio between methane and

trichloroethylene for biodegradation. His model which fitted Haldane kinetics allows to estimate degradation rates depending on the pollutant and methane concentration (Wendlandt et al. 2010). When using single-stage bioreactors, this complex relationship between methane and pollutant concentrations should be considered to obtain optimal results. A way to avoid competitive inhibition problems between substrates, is the use of a multi-stage bioreactor. In these reactors, growth on the primary substrate (methane) and pollutant degradation occur in different reactors. In a typical two-stage bioreactor, the methanotrophs are grown in suspension in a first-stage CSTR while in the second stage a plug flow reactor (Alvarez-Cohen and McCarty 1991) or a hollow fiber reactor (Aziz et al. 1995; Pressman et al. 1999; 2000) is supplemented with the effluent of the first stage reactor and the pollutant contaminated water. However two-stage bioreactors have a complicated configuration that might lead to scale-up problems (Yu 2008). In practice, combining an on-farm biogas installation with a BPS could provide the methane needed for MOB activity making it an inexpensive substrate that can be easily dosed to the system. Combined with the fact that a wide variety of pollutants can be degraded using MOB, using MOB in pesticide biopurification systems seems promising. Despite the advantages of the use of methanotrophs for co-metabolic removal of pollutants, some challenges remain: (I) Competitive inhibition between methane and pollutant is difficult to minimize on a reactor scale (Yu et al., 2008). (II) Low solubility of methane and oxygen in water leads to slow growth and low density cultures (Jiang et al., 2010). (III) Handling of methane is technically difficult due to the risk of explosion when mixed with air. (IV) toxicity of the pollutant towards the MOB. However recently, some facultative methanotrophs have been found that can utilize a variety of carbon substrates such as ethanol or acetate for growth while constitutively expressing MMO regardless of the growth substrate. These bacteria have been found to also be able to degrade a wide range of chlorinated hydrocarbons (Im and Semrau 2011; Yoon et al. 2011). Given this, facultative methanotrophs could be grown on a carbon source such as acetate, while MMO can be completely used for pollutant degradation, eliminating all competitive inhibition problems (Semrau et al. 2011). Nonetheless, toxicity of the pollutant or its degradation products towards these facultative MOB remains possible. Also, the extent

and range of pollutants that can be degraded and the competition with heterotrophs for any added acetate or ethanol and needs to be investigated.

Nevertheless, some problems concerning competitive inhibition between growth substrate and pollutant for the active enzyme as well as toxicity of the pollutant or its intermediates need addressing. Provided these challenges can be overcome, AOB as well as MOB have an enormous potential for use in biodegradation.

5 GUIDELINES TO SECURE MAXIMAL PESTICIDE DEGRADATION EFFICIENCY IN BIOPURIFICATION SYSTEMS

This study permits to propose several guidelines to secure maximal pesticide degradation efficiency in biopurification systems.

- (1) An inoculation source should be used to start up the system. Since pesticide degrading micro-organisms are important to dissipate the pesticides inside a BPS, it is advisable to supplement the system with the right degraders. This should be done at start-up. This approach has several advantages.

First of all, inoculation with a mixed pesticide degrading culture is able to reduce the leaching of more mobile and toxic intermediates. Our results demonstrate that the leaching of the intermediate 3-chloroaniline was reduced by adding a chloropropham degrading biofilm culture to the system (chapter 3).

Secondly, the addition of pesticide degrading bacteria can significantly reduce the amount of time needed to start up biodegradation inside a BPS. In chapter 4 we demonstrated that non-inoculated reactors need a longer start-up period to achieve maximum degradation efficiency. The biomix can be augmented with single pesticide degrading bacteria that were isolated in the lab but our findings suggest that better results can be achieved when mixed pesticide degrading cultures are used as during degradation, significantly less intermediate products are formed in this case. In the system a robust biofilm should be formed that can protect bacteria against several environmental stresses and wash out from the reactor (Flemming 1993). It may also be advantageous to use multi-component systems such as a

microbial consortium that can degrade more than one pollutant ensuring the metabolic diversity and robustness needed for prolonged degradation (Rahman et al. 2002; Diaz 2004). The inoculants can be supplied to the BPS under the form of a biofilm culture grown on carrying materials, which can be easily mixed into the total volume of the BPS at start-up (Fragoeiro and Magan 2008; Bastos and Magan 2009). Residual biomixes from operated BPS, which have repeatedly been exposed to pesticides would be ideal. When such materials are not available, other materials which have been exposed to pesticides such as agricultural soil, can be used.

- (2) Shorter reactors can be used. Our findings described in chapter 5 suggest that the major part of the biodegradation seems to take place in the top part (first 50 cm) of the biofilters. This could make it possible to use shorter biofilter reactors to treat chloropropham waste streams. To prevent leaching of more mobile pesticides from the system, a number of short, 0.5 m high reactors could be stacked on top of each other, guaranteeing a combination of maximum degradation efficiency and retention capacity for more mobile products on a limited amount of space, making this type of bioremediation technique even more feasible.

6 SUGGESTIONS FOR FURTHER RESEARCH

The experiments presented in this work provide an indication about the possible use of pesticide degrading mixed cultures as an inoculation source in the start-up of a new biopurification system using the model component chloropropham. Additional research is necessary to gain insight in the applicability of this method and to further refine this strategy for practical use. Several questions remain to be answered:

- (1) The used model component chloropropham proved to be only moderately recalcitrant. Indeed, our results in chapter 4 show that after a prolonged start-up period compared to the inoculated reactors, the non-inoculated reactors become able to degrade chloropropham and its intermediate 3-chloroaniline. Although only materials that have not been in contact with chloropropham before were used to inoculate the non-inoculated biofilters, these reactors seem to possess the intrinsic

capacity to degrade chloropropham and its intermediate 3-chloroaniline. This means that the genes necessary to degrade chloropropham and 3-chloroaniline are widespread in the present environment. It would therefore be interesting to test the results of this research using more recalcitrant pesticides such as metsulfuron-methyl (half life > 120 days) or metazachlor (half life > 50 days) or more easily degradable pesticide that have very highly persistent degradation products such as chloridazon which forms the N,N-dimethylsulfamide (DMS) (Weber et al., 2008). This metabolite has been shown to form the highly toxic N-nitrosodimethylamine upon ozonation during drinking water treatment (Schmidt and Brauch, 2008). Moreover, a continuous input of persistent pesticides might lead to toxic concentrations in the biomix, resulting in failure of the system.

- (2) Secondly it would be interesting to know how our inoculation approach works when the system is inoculated to treat waste streams containing a variety of pesticides. Also the effect of pesticide adjuvants such as surfactants, oils, compatibility agents, deposition agents, drift control agents and thickeners on biodegradation efficiency and the microbial community should be investigated. It could be that toxicity of one or more pesticides or adjuvants towards the microbial community degrading one or more pesticides can have a negative effect on the overall functioning of the system.
- (3) Thirdly, more mobile pesticides could be tested. Pesticides such as the used model component chloropropham are strongly retained by the biomix which automatically results in a lower breakthrough of the component. More mobile pesticide can migrate quite fast through the system resulting in incomplete degradation of the mother compound or its transformation products. It should be investigated which fluxes can be used to obtain maximum degradation efficiency.
- (4) Our results show that methanotrophs are able to degrade a wide variety of pesticides. The use of these methanotrophs to degrade pesticides in a reactor set-up should be investigated. Some problems can occur regarding competitive inhibition

between methane and the added pesticides as well as low solubility and handling difficulties concerning the use of methane (see above). A possible solution could be the use of facultative methanotrophs which can use ethanol or acetate for growth while constitutively expressing MMO. The use of these facultative methanotrophs to degrade pesticides should be investigated in batch experiments as well as in a reactor set-up.

APPENDICES

1 ABSTRACT

The intensive useage of pesticides to preserve and increase global food production has given rise to increasing concentrations of pesticide residues in surface and groundwater. 40 to 90 % of surface water contamination by pesticides can be attributed to direct losses such as spillages resulting from filling and leakage of the spray equipment, spray leftovers and technical rest volumes in the tank, pump and brooms. Usage of on-farm biopurification systems (BPS) has been recommended to treat the collected pesticide contaminated wastewater. In a BPS the pesticide contaminated wastewater is collected and fed onto a biologically active solid matrix called a biomix which typically consists of straw, soil peat and other organic materials. Microbial communities within a BPS are not always adapted to rapidly and completely degrade specific pollutants or their transformation products. A good approach to solve this problem would be to add the microbial degradation potential needed to remove these recalcitrant pesticides to the system. In the present work, microbiota carrying out metabolic as well as co-metabolic degradation reactions are used to improve the removal of pesticide from contaminated wastewater.

In the first part of this work metabolic reactions are used to obtain the complete mineralization of chloropropham, a potato sprouting suppressant. The metabolic potential to degrade chloropropham was obtained from sludge and soil samples through two different enrichment techniques i.e. planktonic enrichments in shaken liquid medium and biofilm enrichments on two types of solid matrixes (plastic chips and gravel). Biofilm enrichment cultures had a different microbial community composition depending on the presence and type of the added solid matrix during enrichment, and were able to completely mineralize chloropropham with less formation of the toxic intermediate 3-chloroaniline compared to the planktonic cultures. This advantage could be attributed to the biofilm architecture improving nutrient availability and allowing cross-feeding between different species present in the biofilm community. The usage of a chloropropham degrading biofilm consortium as an inoculant for biopurification systems

was tested using pilot scale biopurification systems. Compared to inoculating pure cultures or no inoculation, the inoculation of a microbial biofilm community resulted in reduced leaching of the more mobile and more toxic intermediate 3-chloroaniline at high hydraulic loads. In another experiment, spatial and temporal differences in degradation characteristics and microbial community composition of inoculated and non-inoculated pesticide biopurification systems were investigated. Two biofilters were inoculated with the chloropropham degrading mixed culture, while the other two were not inoculated. Biodegradation rate, size and composition of the microbial community were monitored during 72 days at different biofilter depths. First of all, results showed that inoculation was not necessary to obtain efficient degradation although it shortens the biofilters' start-up period. Secondly, a higher biodegradation rate and chloropropham and 3-chloroaniline degrading microbial community size could be seen in the top part of the inoculated as well as the non inoculated biofilters. Finally, analysis of the microbial community composition shows that no clear spatial stratification of the microbial community could be found in any biofilter. However, the microbial diversity increases over time in all biofilters and on all biofilter depths, suggesting that during the time of the experiment, the biofilters developed a broad carrying capacity in which a genetically very diverse range of chloropropham and 3-chloroaniline degrading species can thrive.

In the second part of this thesis, inocula carrying out cometabolic reactions were used to degrade pesticides. These are reactions that do not sustain the growth of the responsible microorganisms and often lead to the formation of transformation products. In this part of the thesis, we tested whether 14 recalcitrant pesticides could be co-metabolically degraded by methane oxidizing bacteria (MOB). Four compounds i.e. chloropropham, metazachlor, ethofumesate and benalaxyl were successfully removed by MOB activity. Cultures expressing the soluble methane monooxygenase (sMMO) as well as cultures expressing the particulate methane monooxygenase (pMMO) enzyme proved to be able to degrade chloropropham, metazachlor and benalaxyl. However, the degradation efficiency and maximum inhibition concentration of metazachlor was higher for the sMMO expressing cultures compared to the pMMO expressing cultures. This means that copper (Cu^{2+}) regulating the expression of the sMMO and pMMO enzyme seems to be an important factor influencing MOB degradation activity.

In conclusion, the results reported in this thesis show that both the metabolic as the cometabolic strategy have a lot of potential to improve the removal of pesticides from farmyard waste streams.

2 SAMENVATTING

Door het intensieve gebruik van pesticiden worden steeds meer pesticide residuen terug gevonden in oppervlakte- en grondwater en dit in schadelijke concentraties. 40 tot 90 % van de pesticiden die in oppervlaktewater worden gedetecteerd, wordt veroorzaakt door puntverliezen. Puntverliezen zijn het gevolg van contaminatie door morsverliezen bij het vullen van de tanks, lekken van het spuitmateriaal of resten spuitvloeistof in de tanks, pompen en spuitmateriaal. Biologische zuiveringssystemen (BZS) kunnen gebruikt worden om afvalwater dat verontreinigd is met pesticiden te behandelen. In een BZS wordt het gecontamineerde afvalwater eerst verzameld in een buffertank en daarna op een microbiologisch actieve matrix gebracht die bestaat uit stro, aarde, turf en andere organische materialen. De microbiologische gemeenschappen in een BZS zijn echter niet altijd in staat om bepaalde pesticiden en hun transformatieproducten snel en volledig af te breken. Daarom zou het interessant zijn om het metabolisch of co-metabolisch potentieel dat nodig is om bepaalde pesticiden volledig af te breken aan de microbiologische gemeenschap toe te voegen.

In het eerste deel van dit werk wordt gefocust op de metabolische reacties die nodig zijn om de kiemremmer chloorprofam volledig te mineraliseren. Om het metabolische potentieel om chloorprofam volledig af te breken te verkrijgen werden twee verschillende aanrijkingstechnieken gebruikt. Bij de eerste aanrijkingstechniek werden geen materialen aan het voedingsmedium toegevoegd zodat planktonische culturen werden bekomen. Bij een tweede techniek werd aan het medium gravel of plastic dragermateriaal toegevoegd zodat biofilm vormende culturen werden bekomen. De structuur van de microbiële gemeenschap was anders bij de biofilm vormende culturen dan bij de planktonische culturen. Daarnaast was de structuur van de microbiële gemeenschap ook afhankelijk van het type dragermateriaal dat werd toegevoegd (gravel of plastic dragermateriaal). De biofilm vormende culturen konden chloorprofam volledig afbreken en vormden daarenboven minder 3-chlooraniline vergeleken met de planktonische culturen. Dit voordeel was te wijten aan de biofilm architectuur die toeliet om voedingsstoffen en degradatieproducten snel uit te wisselen tussen de verschillende bacteriële species in de biofilm. De chloorprofam degraderende biofilm werd dan ook gebruikt om pilotschaal BZS systemen te inoculeren. Door het gebruik van deze biofilm culturen kon het uitlogen

van het mobielere en meer toxische intermediair 3-chlooraniline verminderd worden vergeleken met het gebruik van pure culturen als inoculant of geen inoculatie. In een ander experiment werden verschillen in degradatiesnelheid en structuur van de microbiële gemeenschap op verschillende dieptes van de BZS in kaart gebracht. Daartoe werden twee biofilters geïnoculeerd met een chloorprofam degraderende biofilm cultuur terwijl twee andere niet werden geïnoculeerd. De afbraaksnelheid van chloorprofam alsook de grootte en samenstelling van de microbiële gemeenschap werd opgevolgd gedurende 72 dagen op verschillende dieptes in de BZS. De resultaten toonden aan dat inoculatie niet nodig was om efficiënte degradatie te bekomen maar dat de opstart periode van de BZS hierdoor wel verkort kon worden. Daarnaast werd een hogere biodegradatiesnelheid en grotere chloorprofam en 3-chlooraniline afbrekende microbiële gemeenschap geobserveerd in het bovenste deel van de van de geïnoculeerde en niet-geïnoculeerde BZS. Analyse van de microbiële gemeenschap toonde aan dat er geen duidelijke verschillen konden gevonden worden op de verschillende dieptes van de BZS. De microbiële diversiteit neemt evenwel toe met de tijd in alle BZS en op alle dieptes. Dit toont aan dat zich gedurende de looptijd van het experiment een fylogenetisch zeer brede verzameling van chloorprofam en 3-chlooraniline afbrekende species ontwikkelt. Dit zorgt er voor dat de microbiële gemeenschap zeer robuust is en bestand tegen stressinvloeden zoals verandering in temperatuur, vochtigheidsgraad, toxische concentraties enz.

In het tweede deel van deze thesis werden cometabolische reacties gebruikt om pesticiden te degraderen. Cometabolische reacties zijn reacties die niet bijdragen tot de groei van de betrokken micro-organismen en vaak de vorming van transformatie producten tot gevolg hebben. Er werden in totaal 14 recalcitrante pesticiden getest om na te gaan of deze cometabolisch konden afgebroken worden door methaan oxiderende bacteriën (MOB). Vier pesticiden i.e. chloorprofam, metazachlor, benalaxyl en ethofumesaat konden worden afgebroken door MOB's. Zowel MOB culturen die het sMMO enzyme als MOB culturen die het pMMO enzyme tot expressie brachten waren in staat chloorprofam, benalaxyl en metazachlor af te breken. De efficiëntie van de degradatie was echter hoger voor de culturen die het sMMO enzyme tot expressie brachten dan bij de culturen die het pMMO enzyme tot expressie brachten. Dit betekent dat koper (Cu^{2+}) dat de expressie van het sMMO en pMMO enzyme regelt een belangrijke factor is.

De resultaten van dit doctoraat tonen dus aan dat zowel de metabolische als cometabolische strategie veel potentieel tonen om aangewend te worden om de verwijdering van pesticiden in een BZS te verbeteren.

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4 CURICULUM VITAE

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Education

2008 – now	Ph.D. in Applied Biological Sciences (LabMET) Doctoral School of Bioscience Engineering – Ghent University
2004 – 2008	Industrial Engineer in Environmental Biotechnology Ghent University
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Professional Career

2008 - now	<ul style="list-style-type: none"> - Scientific collaborator at Laboratory of Microbial Ecology and Technology (LabMET) and Laboratory of Environmental and Biomass Technology - PhD-research: Bioaugmentation strategies for the treatment of pesticide waste streams <p>Promotors: Prof. dr. ir. Nico Boon and Prof. dr. ir. Leen De Gelder</p> <ul style="list-style-type: none"> - Teaching activities: Practical exercises: “Microbiology”, “Environmental Technology”, “Biology” and “Applied Biokatalysis”
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- Tutor of 6 master thesis students: 5 industrial engineer and 1 bio-engineer

Publications

All publications

Hennebel, T., Verhagen, P., Simoen, H., De Gusseme, B., Vlaeminck, S., Boon, N. and Verstraete, W. (2009) Remediation of trichloroethylene by bio-precipitated and encapsulated palladium nanoparticles in a fixed bed reactor. *Chemosphere* **79**, 1221-1225.

Hennebel, T., De Corte, S., Vanhaecke, L., Vanherck, K., Forrez, I., De Gusseme, B., Verhagen, P., Verbeken, K., Van der Bruggen B., Vankelecam, I. and Verstraete, W. (2010) *Removal of diatrizoate with catalytically active membranes incorporating microbially produced palladium nanoparticles*. *Water Research* **44**, 1498-1506.

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Hennebel, T., Simoen, H., Verhagen, P., De Windt, W., Dick, J., Weise, C., Pietschner, F., Boon, N. and Verstraete, W. Biocatalytic dechlorination of hexachlorocyclohexane by immobilized bio-Pd in a pilot scale fluidized bed reactor. (2011) *Environmental Chemistry Letters* **9**, 417-422.

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Verhagen, P., Destino, C., Boon, N. and De Gelder, L. (2015) Spatial heterogeneity in degradation characteristics and microbial community composition of pesticide biopurification systems. *Journal of Applied Microbiology* **118**, 368-378.

Verhagen, P., Benner, J., Claes, L., De Gelder, L. and Boon, N. (submitted) Pesticide degradation using methanotrophs: influence of pesticide concentration and the microbial community.

Other publications

Verhagen, P., De Gelder, L. and Boon, N. (2010) Biofilm based bioaugmentation strategies for the treatment of pesticide waste streams. (2010) *Pesticides and related organic micropollutants in the environment*, 6th European conference, abstracts

Verhagen, P., De Gelder, L. and Boon, N. (2012) Biofilm based bioaugmentation

strategies for the treatment of pesticide waste streams. *Microbial Ecology*, 14th International symposium, abstracts

Verhagen, P., De Gelder, L. and Boon, N. (2014) Spatial and temporal differences in degradation characteristics and microbial community composition of pesticide biopurification systems. *Communications in applied biological sciences* **79**, 173-178.

Presentations

Biofilm based bioaugmentation strategies for the treatment of pesticide waste streams, 6th European conference on pesticides and related organic micropollutants in the environment, 7-10 september 2010, Matera, Italy (oral and poster presentation)

Biofilm based bioaugmentation strategies for the treatment of pesticide waste streams, ISME conference, 19-24 augustus 2012, Copenhagen, Denmark (poster presentation)

Spatial and temporal differences in degradation characteristics and microbial community composition of pesticide biopurification systems, 19th national symposium on applied biological sciences, 7 februari 2014, Gembloux, Belgium (oral presentation)

Spatial and temporal differences in degradation characteristics and microbial community composition of pesticide biopurification systems, 16th international biodeterioration and biodegradation symposium, 3-5 september 2014, Lodz, Polen (poster presentation)

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